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Microbial communities structure of the Japan Trench cold-seeps determined by lipid analysis

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Microbial communities structure of the Japan Trench cold-seeps determined by lipid analysis

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1. INTRODUCTION

1.1 Seafloor Cold Seeps Environment

Cold Seeps have been discovered in the active convergent continental margins (e.g., the Japan Trench, the Nankai Trough, the Barbados Trench, off the coast of Oregon, off the coast of Peru, the Mediterranean Sea), passive continental margins (e.g., the North Sea, the Gulf of Mexico, the Arctic Basin) and shallow intertidal environments (e.g., the Danish coast) (Figure 1) (Kulm et al., 1986; Ohta and Laubier, 1987; Sibuet et al., 1988; Schmaljohann et al., 1990; Aharon 1994, 2000; Dando et al., 1994; Kato et al., 1999; Zhang and Lanoil, 2004). Seafloor cold seeps are where chemically-enriched, oxygen-depleted fluid releases into the ocean by means of diffusion through sediments (Aharon, 2000, Leveille and Juniper, 2003). Chemosynthetic fauna, bacterial mats, gas hydrates, bubbling methane vents and massive carbonate deposits are common cold seep features (Joye et al., 2004; Orphan et al., 2004). At the subduction zone, where the oceanic plates are being subducted beneath continents, large amount of sediments overlying the continental plate are scraped and compressed. Since these sediments have a high content of pore fluids, in the course of subduction, the fluids are squeezed and diffused through sediment or flow along major faults (Herzig and Hannington, 2000; Leveille and Juniper, 2003). Also, the seeping fluids are often enriched in methane, which leads to very high metabolic rates, and contributes to the emergence of dense microbial communities (Suess et al., 1999; Boetius et al., 2000; Tryon and Brown, 2001).

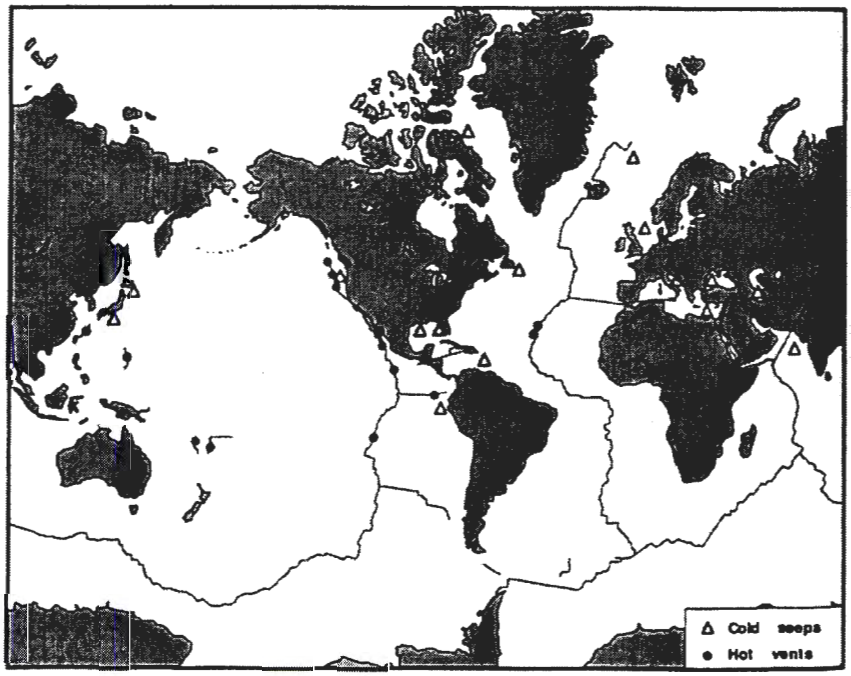

Figure 1. World distribution of cold seeps (Δ) and hot vents (●) on the ocean floor (Aharon, 2000).

Chemosynthetic communities in cold seeps are large assembles of marine organisms that depend on a chemoautotrophic food chain which starts with the microbially-mediated oxidation of reduced compounds (Elvert, 2000). Some of these chemoautotrophic microorganisms are living as symbionts with the chemosynthetic macrofauna found in the area. The macrofauna normally demonstrate a patchy distribution on the seafloor where reducing compounds are abundant (Barry et al., 1997; Goffredi and Barry, 2002). They also impact the physical and geochemical environment in the area by burrowing into and ventilating the sediment (Tryon and Brown, 2001).

At seafloor cold seeps, these heterotrophic invertebrates develop a mutual symbiosis with their symbiotic microorganisms, i.e., an association between two organisms which both

the hosts and symbionts derive some degree of benefit. Vestimentiferans, mytilids, epifaunal vesicomyid clams, and infaunal lucinid/thyasirid clams are four common chemosynthetic community types (MacDonald et al., 1989). These deep-sea organisms are unable to produce their own food and do not have digestive systems, so they rely upon symbiotic microorganisms living inside their tissue as a source of nutrients. The symbionts supply food for the hosts and consume toxic substances that might poison the host's aerobic metabolism. On the other hand, the hosts also provide symbionts protection and attachment to gain simultaneous access to methane, sulfide, or oxygen (Lee, 1999). For example, in the Gulf of Mexico cold-seeps, chemosynthetic mytilid mussel beds are usually correlated with the simultaneous availability of methane and oxygen. The mussels provide methane and oxygen to the methanotrophic symbiotic bacteria present in their gills tissue (Fang et al., 1993). On the other hand, they rely on the metabolic intermediates synthesized by their endosymbiotic bacteria as their food source (Jahnke et al., 1995).

1.2. Chemosynthetic Microbial Communities and Biogeochemical Processes

The cold seeps' major characteristic is comparable to that of hydrothermal vents; while relatively hot fluids release through hydrothermal vent, cold seeps are characterized by cold temperature fluids (close to the surrounding seawater temperature, 2-3°C). The slow seeping fluids diffuse from the subsurface and migrate along the faults and factures and approach the seafloor (Leveille and Juniper, 2003). The seeping fluids, with high concentrations of methane or sulfide, are needed for chemosynthesis and flourish lives in the deep sea. Both methane and sulfide are utilized by microorganisms to gain energy from the reduction of sulfate and the oxidation of methane, or the so-called anaerobic oxidation of

methane (AOM) (Elvert et al., 2000). These deep-sea microorganisms are a vital and unique part of the global ecosystem, because they consume large amounts of methane produced along hydrothermal vents and cold seeps. Without these microorganisms, the methane would have an impact on the atmosphere and overall environment (Valentine, 2002). Unlike photosynthetic cyanobacteria at the earth's surface, some deep-sea microorganisms are chemolithoautotrophic. They make food by using chemical energy instead of energy from the Sun. Chemolithoautotrophic microorganisms can work independent of surface life, but most of them still depend on the dissolved oxygen and/or reduced carbon compounds that are generated by the photosynthesis in photic zone (Joye et al., 2004).

Lines of evidence proved that AOM in cold seeps has a significant role on the global methane cycling (Valentine, 2002). Large amounts of methane are produced in cold seeps, but only 2% of annual global methane input to the atmosphere is coming from the marine environment (Reeburgh, 1996). AOM consumes nearly 90% of the methane generated in the cold seeps (Hinrichs et al., 2000). Although none of the organisms that can oxidize methane anaerobically has ever been isolated, biogeochemical evidences suggest that AOM requires a complex consortium of microorganisms working in syntrophic partnership in cold seep ecosystems (Elvert et al., 2000). The overall AOM process involves a transfer of electrons from methane to sulfate and is probably mediated by at least two groups of organisms, including methane-oxidizing archaea and sulfate-reducing bacteria (Hoehler et al., 1994; Hinrichs et al., 1999). At seeps, both CH_4 and H_2S provide primary energy for microbial chemosynthesis. Energy production is a two-part process. First, the reducing substances are oxidized either by free-living microbes in the water column and within the sediment, by the attached microbes (bacterial mat, such as *Beggiatoa*), or by the symbionts living within

macrofauna (Aharon, 2000). Methane-oxidizing archaea or methanogens, running in reverse, oxidize methane to carbon dioxide and hydrogen gas, or into acetate (Elvert, 2003; Leveille and Juniper, 2003). Then, sulfate-reducing bacteria (SRB) reduce sulfate while oxidizing hydrogen and acetate, two products of methane oxidation (Leveille and Juniper, 2003, Hoehler et al., 1994). The overall syntrophic process is called anaerobic oxidation of methane (AOM).

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$$

1.3 Determining Microbial Community Structure Using Lipid Biomarkers

Biomarkers, also called biological markers or molecular fossils, have a wide range of applications in biogeochemistry. They are often used to describe and quantify complex microbial communities in environmental samples. Biomarkers are organic molecules presented in the geosphere with indisputable biological origins (Eglinton and Calvin, 1967). Biomarkers must have good chemical stability and structure that can be unambiguously linked to the source organisms. Based on their biosynthetic pathways, lipid biomarkers can be classified into two types: acetogenic and isoprenoid lipids (Figure 2). Acetogenic lipids are also termed linear or *n*-alkyl lipids (Figure 3). Their linear skeletons are derived from the acetate biosynthetic pathway. They include simple fatty acids containing no hydrolysable linkage and complex lipids containing one or more ether and ester bonds (Hayes, 2001). Isoprenoid lipids have a wide variety of structures. They are based on the isopropene unit, but there are variations on (1) the connection between isoprene units and (2) the lipids' cyclization. Isoprenoids are biosynthesized through the mevalonate or the methylerythritol-

phosphate pathways (Hayes, 2001). These lipids include acyclic and cyclic isoprenoids (e.g., phytol) and polycyclic isoprenoids (e.g., hopanoids and hopane -- a derivative from hopanoids) (Figure 4).

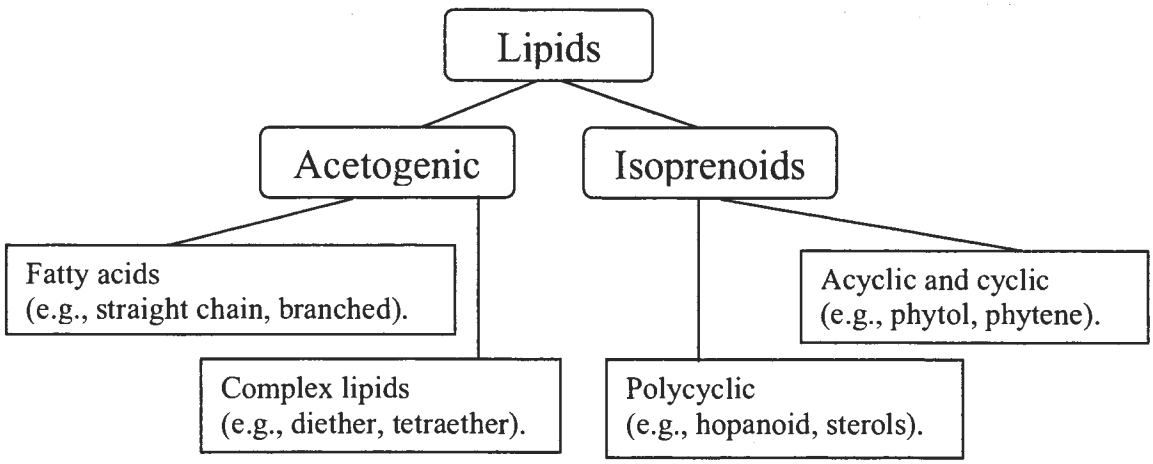

Figure 2. The classification of lipids.

Both acetogenic and isoprenoid lipids can be found in microbial lipids. Microbial lipid biomarkers are among the most abundant compounds present in both marine and terrestrial, and in both modern and ancient sediments (Pancost and Damste, 2003). Thanks to their diverse functionalities and carbon skeletons, (Pancost and Damste, 2003) these lipid biomarkers provide information on ancient environmental conditions and prokaryotic processes in complex modern ecosystems. However, the effectiveness of a lipid biomarker depends on whether a given lipid is diagnostic for a specific organism (Pancost and Damste, 2003). In the following sections, some of the major lipid classes used in this project will be discussed.

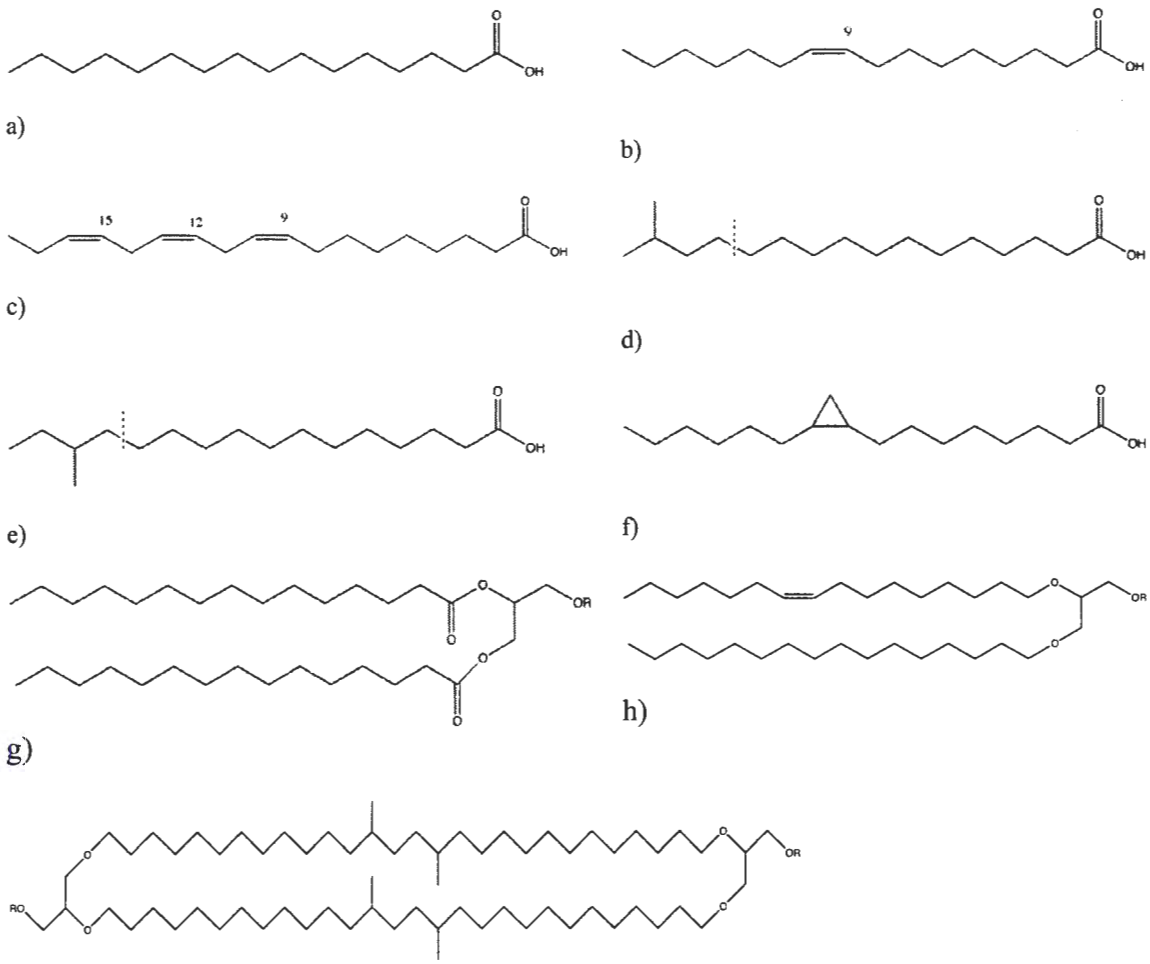

Figure 3. Representative acetogenic lipids. The polar head group is represented by R in the shorthand structures. a) a saturated fatty acid, C16:0; b) a monounsaturated fatty acid, C16:1Δ^{9}; c) a polyunsaturated fatty acid, C16:3$\Delta^{9,12,15}$; d) branching fatty acid (iso); e) branching fatty acid (anteiso); f) a cyclopropyl fatty acid; g) phospholipids, a major component of biological membrane; h) glycerol diethers, a component of archaeal membranes, the nonpolar chains are joined to a glycerol backbone by ether rather than ester linkages; i) diglycerol tetraether, another archaeal membrane lipid.

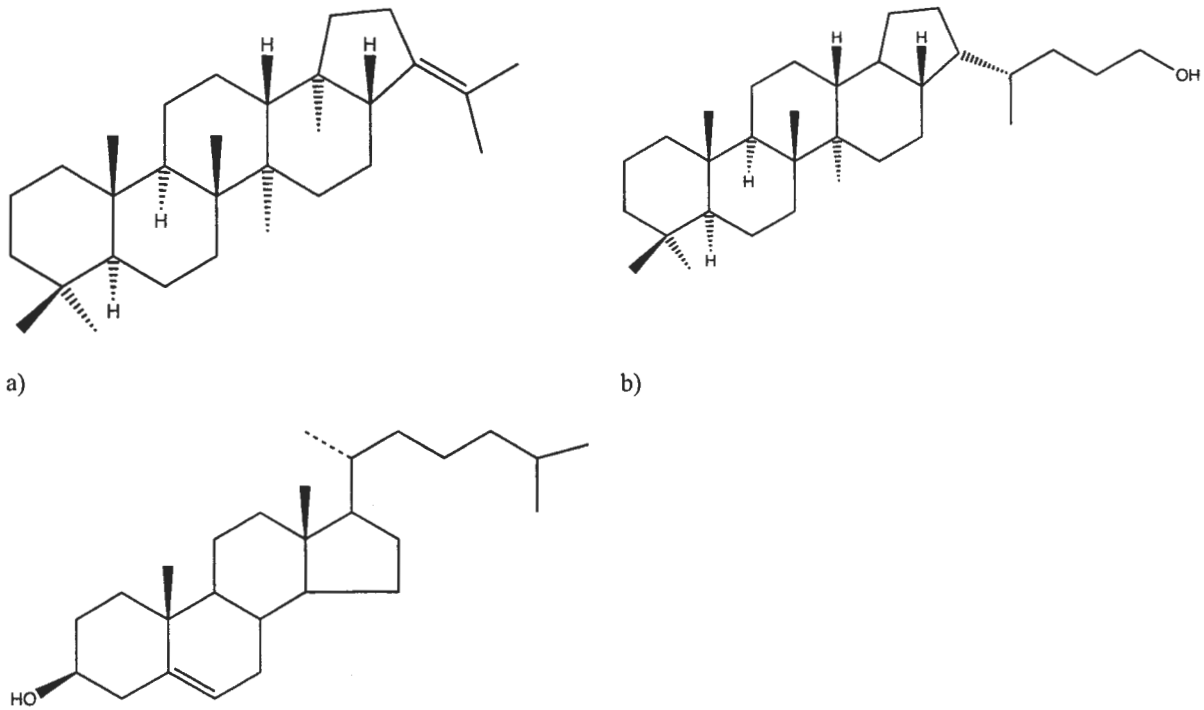

Figure 4. Examples of polycyclic lipids: a) Hop-21-ene (a pentacyclic triterpenoid lipid), the hydrocarbon tail is linked to the five linked carbon rings, and a double bond is present at the carbon 21; b) Bishomohopan-32-ol (pentacyclic triterpenoid lipids), a hydrocarbon tail with hydroxyl group is attached to the five linked carbon rings; c) Cholesterol (tetracyclic triterpenoid lipids), a hydrocarbon tail is linked to the four linked carbon rings, and a hydroxyl group is attached at the other end.

1.3.1 Phospholipid Fatty Acids (PLFA)

Phospholipids, glycolipids, and neutral lipids are the three major kinds of membrane lipids. In particular, phospholipid fatty acid analysis has proved to be useful in bacterial phylogenetic and taxonomic classification (Lechevalier, 1977). It also provides a more reliable basis for making inferences about community composition (White et al., 1979;

Guckert et al., 1985; Green and Scow, 1999). A list of microorganism and their characteristic phospholipid fatty acid biomarkers is shown in Table 1. Phospholipids are made up of a 3-carbon glycerol molecule, a polar head group, and two fatty acids esterified to the glycerol backbone (Figure 3g). Phospholipids constitute a part of every cellular membrane and form a constant proportion of the membrane of various microorganisms (White et al., 1979). They are actively metabolized during the growth of bacterial monocultures (White and Tucker, 1969) and have a relatively rapid turnover in sediments (White et al., 1979) and in soils (Tollefson and McKercher, 1983). Therefore, an estimate of the microbial biomass measurement *in situ* can be obtained through simple lipid extraction of sediment (White, 1988). Furthermore, a sensitive analytical PLFA analysis only requires a small quantity of environmental sample (e.g., 20-100 mg sediment) (Fang and Findlay, 1996).

Table 1. Fatty acid biomarkers of different functional groups of microorganisms.

Organism	PLFA biomarker	Reference
Aerobic prokaryotes and eukaryotes	C16:1$^{\Delta 11}$, C16:1$^{\Delta 9}$c, C17:1$^{\Delta 11}$, C17:1$^{\Delta 8}$, C18:1$^{\Delta 11}$c, C18:1$^{\Delta 9}$, C18:2$^{\Delta 12}$	Findlay and Dobb et al., 1993
Gram-positive bacteria	*a*C15:0, *i*C15:0, *i*C16:0, 10Me-C17:0	Findlay and Dobb et al., 1993; Kaneda, 1991; White et al., 1996
Gram-negative bacteria	C17:0*cy*, C19:0*cy*, C18:1$^{\Delta 7}$c, 16:1$^{\Delta 9}$, 18:1$^{\Delta 11}$, 18:1$^{\Delta 9}$	Findlay et al., 1990
SRB and other anaerobes	C16:0, 10Me-C16:0, *i*C17:0, *a*C17:0, C17:0*cy*, C17:0, C18:0, C19:0*cy*	Findlay and Dobb, 1993
Desulfobacter	10Me-C16:0, C18:0$^{\Delta 10,11}$*cy*	Dowling et al., 1986; Kuever et al., 2001; Rutters et al., 2002
Desulfobulbus	C17:1$^{\Delta 11}$c, C15:1	Parker et al., 1982; Taylor and Parkes, 1983; Parkes and Calder 1985
Desulfotomaculum	*i*C15:0, *a*C15:0	Liu et al., 1997; Kuever et al., 1999; Chang et al., 2001; Ueki and Suto, 1979
Desulfomonile tiedjei	C16:1$^{\Delta 9}$c, C17:0*cy* and hydroxyl fatty acids in LPS	Ringelberg et al., 1994
Desulfobacterium	10Me-C16:0, C17:1$^{\Delta 11}$c, C16:1$^{\Delta 9}$c	Kuever et al., 2001; van der Maarel et al., 1996
Desulfococcus multivorans	*i*C15:0, *a*C15:0, C16:1$^{\Delta 9}$, *a*C17:1$^{\Delta 10}$, *i*C17:1$^{\Delta 10}$, C17:1$^{\Delta 11}$, C17:1$^{\Delta 9}$	Rutters et al., 2002
Desulfomicrobium sp. strain SAL	C16:1$^{\Delta 9}$, iC17:1$^{\Delta 10}$, *a*C17:1$^{\Delta 10}$, C17:1$^{\Delta 11}$, C17:1$^{\Delta 9}$, C18:1$^{\Delta 11}$	Rutters et al., 2002
Type I methanotroph	C16:1$^{\Delta 8}$c, C16:1$^{\Delta 10}$c, C16:1$^{\Delta 9}$c, C16:1$^{\Delta 9}$t, C16:1$^{\Delta 11}$c, C16:1$^{\Delta 11}$t, C16:1$^{\Delta 7}$c	Makula, 1978; Nichols et al., 1985; Bowman et al., 1993; Guckert et al., 1991
Type II methanotroph	C18:1$^{\Delta 10}$c, C18:1$^{\Delta 10}$t, C18:1$^{\Delta 12}$c, C18:1$^{\Delta 11}$c, C18:1$^{\Delta 11}$t	Makula, 1978; Nickols et al., 1985; Nichols et al., 1987
Sulfur oxidizer	C16:1$^{\Delta 9}$,C18:1$^{\Delta 11}$	Larkin, 1980; Katayama-Fujimura et al., 1982; Jannasch, 1985; Jannasch et al., 1989; Guezennec and Fiala-Medioni, 1996
Thiomicrospira sp.	C14:1, *i*C15:0, *a*C15:0, C16:1, C18:1	Grimalt et al., 1992; Knittel et al., 2005
Beggatoia and *Thioploca spp*	C18:1$^{\Delta 11}$c, C16:1$^{\Delta 9}$	McCaffrey et al., 1989; Grants, 1991

1.3.2 Hopanoids

Hopanoids are pentacyclic triterpenoid lipids found in bacterial membrane (Kannenberg and Poralla, 1999). They regulate and rigidify the bacterial membrane that is similar to the function of sterols in Eukarya (Summons et al., 1999). They have been detected in 30% of all bacteria investigated, including a wide range of Gram-negative and Gram-positive bacteria. They occur regularly in aerobic bacteria, such as cyanobacteria, methanotrophs, heterotrophs (Rohmer et al., 1992), but not in anaerobic microorganism, including archaea and sulfate-reducing bacteria (Ourisson and Rohmer, 1992). However, recent research indicates that hopanoids also occur in two species of strictly anaerobically bacteria, i.e., *Geobacter metallireducens* and *Geobacter sulfurreducens* (Kannenberg et al., 2005).

Geohopanoids, the buried and altered biohopanoids in the sediments, are among the most abundant organic molecules in the sediments and have been found in a variety of environments including cold seep setting (e.g., the Black Sea; Thiel. et al., 2003). Geohopanoids can serve as biomarkers, and they help pinpoint their biological origins, (Ourisson and Albrecht, 1992) as they represent the oldest chemical fossils to which a discrete biological source can be attributed (Summons et al., 1999). Particularly, methylhopanoids contain very useful source-diagnostic information. For example, 3β-methylbacteriohopanoids and 2β-methylbacteriohopanoids are specific to methane-oxidizing bacteria (Zundel and Rohmer, 1985) and cyanobacteria (Summons et al., 1999), respectively.

1.4 Project Scope

Studies of microbial diversity associated with deep-sea environments have long been restricted to the microscopic description of samples and the isolation of a limited number of microorganisms. These traditional techniques are selective and not quantitative (Vestal and White, 1989; White et al., 1997); most microorganisms in the environment are viable but not culturable (Amann et al., 1995; McCarthy and Murray, 1996; Pace, 1996; White et al., 1997). Viable counts of bacteria in environmental samples determined with classical methods represent only a small fraction (0.1% to 1%) of the active microbial community (Olsen and Bakken, 1987; White et al., 1997). Determining microbial community composition by molecular finger-printing using small subunit ribosomal RNA and by biochemical analysis based on phospholipid fatty acid composition are two methods that can overcome the selective bias of the traditional cultivation techniques. Even though molecular based techniques provide useful information on identifying microorganisms, it does not provide us quantitative information about the microbial communities. Although, rRNA gene sequences (rDNA) can be easily amplified from bulk DNA recovered from sediment, it is not known whether the signatures obtained reflected the present living cells or were a mixed community composed both of living and dead cells. In studies of microbial community structure of cold seep areas, no endosymbiotic bacteria have been isolated to date. The current method of estimating bacterial cell density within bacteriocytes of the invertebrate hosts based on microscopic enumeration is difficult and inaccurate. (Balkwill et al., 1988)

The lipid-based approach is often alternatively used for estimating microbial diversity in sediment. Total biomass can be measured by the summation of the mass of all detectable phospholipid ester-linked fatty acids (White et al., 1979). Quantitative biochemical analysis has advantages over classical microscopic enumeration and morphological examinations in

determining microbial biomass, community structure, and metabolic activity (White et al., 1979). Phospholipid ester-linked fatty acids represent the viable cellular biomass and can be extracted quantitatively (Fang and Findlay, 1996). So, the measurement of PLFA provides a quantitative estimate of microbial biomass in cold seeps.

The overall objectives of this project are: (1) to determine the microbial biomass and community structure in sediments from two chemosynthetic communities dominated by the *Calyptogena phaseoliformis* and *Maorithyas hadalis* communities; (2) to identify the symbionts living inside the *Maorithyas hadalis* gill tissue and compare them with the free-living microbes in the sediment; and (3) to determine the biogeochemical cycle of methane and sulfur in the Japan Trench cold seeps involving the free-living and symbiotic bacteria and chemosynthetic fauna.

2. MATERIALS AND METHODS

2.1 The Japan Trench Cold-seeps

The Japan Trench cold-seeps are the deepest seafloor cold seep environment found on Earth (Kato et al., 1999). Located in the western North Pacific Ocean, it is where the Pacific oceanic plate is subducted beneath Eurasia (von Huene et al., 1982; Deyhle et al., 2004). The Japan Trench is located on the southeastern side of Japan and parallels the coast of Honshu Island. Its depth is about 7000 to 8000 m. It is one of a series of depressions stretching south from the Kuril Trench and the Bonin Trench to the Mariana Trench (Figure 5).

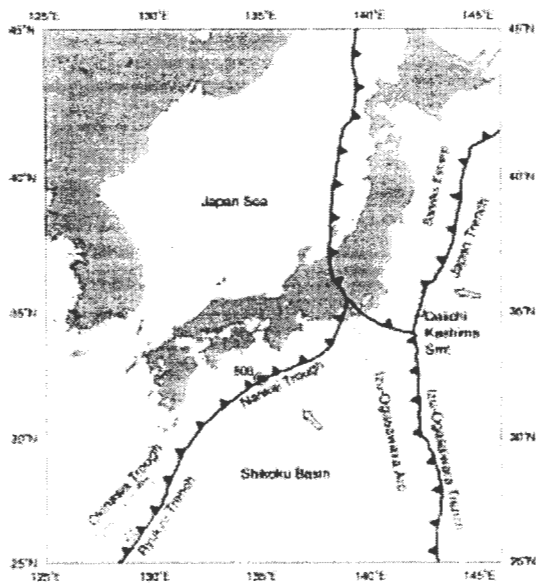

Figure 5. Map of Japan Trench area (Kobayashi, 2002)

Sediment and clam samples for this project were collected from two chemosynthetic bivalve communities dominated by *Maorithyas hadalis* and *Calyptogena phaseoliformis*. The *Calyptogena phaseoliformis* and *Maorithyas hadalis* communities were discovered near the bottom of the Japan Trench in 1999 (Fujikura et al., 1999; Kato et al., 1999). *Calyptogena sp.* is a vesicomyid clam that is typically found in cold-seep areas (e.g., the Nankai Trough; Li at el., 1999; Kato et al., 1999). This vesicomyid clam relies on chemosynthetic bacteria, sulfur-oxidizing endosymbionts, that inhabit their gill surfaces and support its growth (Paull et al., 1984; Kennicutt et al., 1985, Li et al., 1999). Evidence shows that the microbial sulfate reduction occurs within the sediment just beneath the *Calyptogena* community (Masuzawa et al., 1992; Li et al., 1999; Kato et al., 1999). The hydrogen sulfide produced is oxidized by the endosymbiotic sulfur-oxidizing bacteria in the gills of *Calyptogena* (Masuzawa et al., 1992; Li et al., 1999). The Japan Trench cold-seeps are dominated by the *Calyptogena phaseoliformis* communities; however, in the deeper areas where *Maorithyas hadalis* is present, the *Calyptogena phaseoliformis* communities were excluded (Fujikura et al., 1999).

The *Maorithyas hadalis* bacterial symbiosis at the Japan Trench (7326 m) is the deepest marine chemosynthetic symbiosis ever recorded (Fujikura et al., 1999). The strong hydrogen sulfide odor from the clam's soft parts and the surrounding sediment as well as the locally dark gray to black color of the sediment surrounding their colony indicate that *Maorithyas hadalis* is living in an anoxic environment with high sulfide concentrations (Fujikura et al., 1999). *Maorithyas hadalis* is a thyasirid clam that contains endosymbiotic coccoid bacteria within its gill tissue (Fujikura et al., 1999; Fujiwara et al., 2001). Unlike most symbionts of thyasirid clams, which have been reported to occur extracellularly, the symbionts of *Maorithyas hadalis* occur intracellularly (Fujikura et al., 1999; Fujiwara et al.,

2001). Two types of intracellular symbionts were discovered from their gill tissue. Symbiont Type I is phylogenetically affiliated with the thioautotrophic symbionts of vesicomyid clams and mussels from deep-sea hydrothermal vents and cold seeps near the surface of the gill. Symbiont Type II is phylogenetically different from any other symbiotic or free-living bacteria and is more concentrated in the inner part of gill (Fujiwara et al., 2001).

2.2 Sample Collection

Four sediment and two clam tissue samples from the Japan Trench (Table 2) were collected and freeze-dried by the Japan Agency for Marine-Earth Science and Technology (JAMSTEC). C-018, C-025 and B-74 were collected from the *Maorithyas* community at depths of 7337 m, 7434 m and 7321 m, respectively. 374-2 and C-022 were collected from the *Calyptogena* community at depths of 6361 m and 5791 m, respectively. *Maorithyas hadalis* clams were collected at a depth of 7336 m and the gill and leg tissue were obtained from dissection in the lab.

Table 2. Description of sediment and clams samples

Sample	Depth	Location	Description
C-018	7337	40 02.81N, 144 16.60E	*Maorithyas* sediments
B-74	7321	40 02.73N, 144 16.45E	*Maorithyas* sediments
374-2	6361	40 06.74N, 144 11.08E	*Calyptogena* sediments
C-022	5791	40 06.84N, 144 10.24E	*Calyptogena* sediments
Gill	7336	40 02.82N, 144 16.60E	*Maorithyas hadalis*
Leg	7336	40 02.82N, 144 16.60E	*Maorithyas hadalis*

2.3 Materials

Silicic acid column and optimal grade solvents (dichloromethane (DCM), acetone, chloroform, hexane, and methanol) were obtained from Fisher Scientific (Pittsburgh, PA). All glassware was washed with phosphate-free detergent, rinsed with distilled and deionized water and air-dried. The glassware was combusted in an oven at 450°C for 48 h and rinsed with DCM just prior to use.

2.4 Lipid Extraction and Fractionation

The analytical scheme for lipid analysis is shown in Figure 6. Total lipids were extracted by the Bligh and Dyer method (Bligh and Dyer, 1959; White et al., 1979) as modified by Fang and Findlay (1996). A solvent mixture of 6 mL methanol, 3 mL DCM and 2 mL phosphate buffer (potassium phosphate, dibasic, 50 mM, pH 7.4) was added to a 15-mL test tube fitted with PTFE-lined screw cap. The freeze-dried sediment samples were weighed and added to the extraction tube and vortexed. The extraction tubes were allowed to sit overnight in darkness at 4°C. The sediment residues were filtered using glass fiber filters (Fisher Scientific, Pittsburgh, PA). The lipids were partitioned from other biological components by adding 3 mL DCM and 3 mL water to the solvent, such that the final ratio of DCM-methanol-water was 1:1:0.9. The separated phases were allowed to stand 24 hours and the top aqueous phase was removed. The bottom phase containing the total lipids was collected.

The extracted lipids were dried and redissolved in hexane:DCM (7:3, v/v) mixture. Total lipids were separated into four different lipid classes using miniature columns (Fisher,

Pittsburgh, PA) containing 100 mg silicic acid. Columns were conditioned before the addition of the sample by flushing them sequentially with methanol (3 mL), chloroform (3 mL), acetone (3 mL) and hexane (3 mL). *n*-Alkanes, neutral lipids, glycolipids, and phospholipids were collected by sequential elution with 5-mL aliquots of hexane, chloroform, acetone, and methanol, respectively (Figure 6). The fractions were collected in test tubes fitted with PTFE-lined screw caps and dried under a stream of nitrogen. The *n*-alkanes fraction was quantified using gas chromatography/mass spectrometry without further purification.

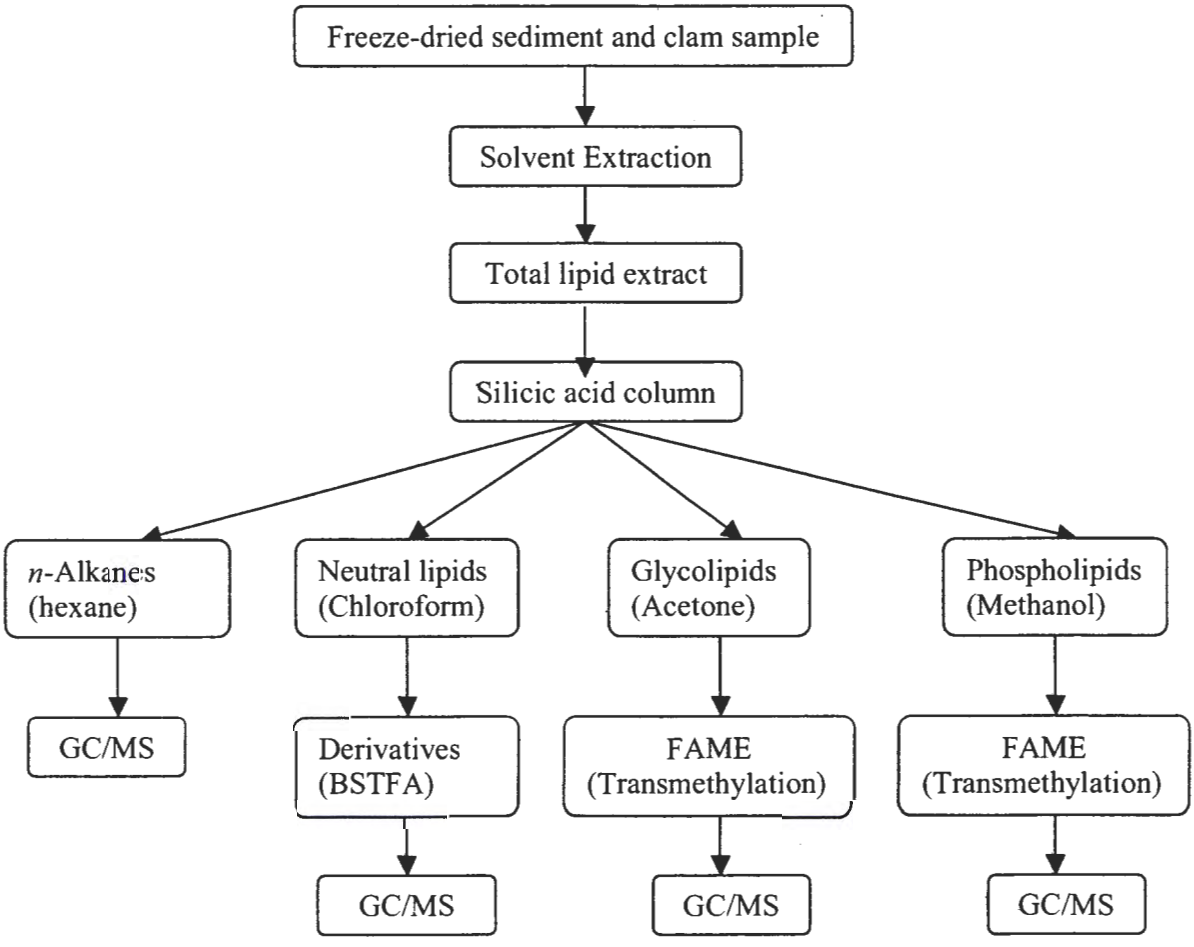

Figure 6. Analytical scheme for lipid extraction, separation and analysis

2.5 Production of Fatty Acid Methyl Esters and Determination of Fatty Acid Double Bond Position

Ester-linked phospholipid fatty acids were subjected to a mild alkaline trans-methylation procedure to produce fatty acid methyl esters (FAME) (Fang and Findlay, 1996). The phospholipid fraction was dried under nitrogen gas and dissolved in 0.6 mL of methanol. One third of the phospholipids were transferred to a test tube fitted with a PTFE-lined screw cap and the remaining (0.4 mL) phospholipids were reserved for future analysis. Then, methanol (0.05 mL), toluene (0.25 mL) and 0.2N KOH in methanol (0.5 mL) were added to the fraction. The solution was vortexed and heated at 37°C for 15 min. The reaction mixture was then cooled to room temperature and 0.5 mL of 0.2 N acetic acid was added. The addition of 2 mL of DCM and 2 mL deionized water separated the mixture into two phases. The bottom organic phase containing the FAMEs was collected, dried and resuspended in 300 μL of DCM. A method blank was extracted with samples and was assumed to be free from contamination if the chromatograms of the blanks contained no peaks.

Double bond position and geometry of monounsaturated fatty acids were determined by using the method described by Dunkelblum et al. (1985). Derivatization reactions were performed in a 2 mL vials with Telfon-lined screw caps. FAMEs in hexane were treated with 100 μL dimethyldisulfide (DMDS) and two drops of iodine solution in diethyl ether (6%). The reaction mixture was kept at 40°C oven for 24 h, cooled, and diluted with 200 μL hexane. The hexane solution containing the DMDS adducts of fatty acids were dried and concentrated with a stream of nitrogen to a small volume (20 μL).

2.6 Glycolipids

Glycolipid fractions were treated with the same mild alkaline trans-methylation procedure as described for the phospholipid fraction to produce fatty acid methyl esters from glycolipids.

2.7 Neutral Lipids

The neutral lipid fraction was dried under nitrogen and treated with 60 μL BSTFA (N,O-bis(tri-methylsilyl)trifluoroacetamide) (Supelco, St Louis, MO). Then, the lipids were heated at 75°C for 1 h. The cooled lipids were dried under nitrogen and dissolved in 300 μL DCM.

2.8 Gas Chromatography/Mass Spectrometry (GC/MS)

Samples of PLFA, *n*-alkanes, neutral lipids, GLFA and DMDS adducts of fatty acids were analyzed on an Agilent 6890N GC, interfaced with an Agilent 5973 mass selective detector. The analytical separation of the compounds was accomplished using a 30 m x 0.25 mm i.d. DB-5 MS fused-silica capillary column (Agilent, Palo Alto, CA). The oven temperature was programmed from 50 to 140°C at 30°C/min, then to 310°C at 5°C/min, and held at 310°C for 20 min. Individual compounds were identified from their mass spectra by comparison with standard or published spectra and from their relative retention times. Response factors were obtained for each compound using duplicate injections of quantitative standards at five different concentration levels. Concentrations of individual compounds

were obtained based on the GC/MS response relative to that of an internal standard (C18:0 fatty acid ethyl ester) and reported as $\mu g\ g^{-1}$ dry weight of sediment.

Selective ion monitoring was employed for the FAME samples. A full scan run was performed to confirm the selected ion monitoring (SIM) analysis results. As with full scan runs, the concentration of each compound detected in SIM mode was calculated and reported as $\mu g\ g^{-1}$ dry weight of sediment.

2.9 Nomenclature

A shorthand nomenclature of two numbers separated by a colon is used to describe fatty acids x:y. The first number indicates the length of the carbon chain. The second number after the colon indicates the degree of saturation (the number of double bonds). The position of the double bond is defined by the symbol (Δ) followed by the number of carbons from the carboxyl end of the fatty acids. The prefixes *i* and *a* refer to *iso* and *anteiso* methyl branching, respectively. Cyclopropyl fatty acids were designed as *cy* with the ring position in parenthesis relative to the carboxyl end. The geometry of the double bonds is indicated by *cis* and *trans*. The 10-methyl-branched fatty acids are indicated by the prefix 10Me.

3. RESULTS

3.1. Phospholipid Fatty Acid Profiles

3.1.1. PLFA Profiles of Sediments

The phospholipid ester-linked fatty acid profiles of sediment samples are shown in Tables 3, 4 and Figure 11, and the total ion chromatograms for four sediment samples are shown in Figures 7, 8, 9 and 10. For the *Maorithyas* sediment, a total of 28 fatty acids were detected, ranging from 12 to 22 carbons in chain length. For the *Calyptogena* sediment, a total of 21 fatty acids were detected, ranging from 14 to 20 carbons. Sediment PLFA profiles of both communities include saturated (SAFA), monounsaturated (MUFA), cyclopropane (CYFA), 10-methyl-branched (10MBFA), and terminal methyl-branched (TBFA) fatty acids. Polyunsaturated fatty acids (PUFA) were only detected from the *Maorithyas* sediments, including C18:2, C20:5 and C22:6. All phospholipid profiles were dominated by SAFA (50% of total for *Maorithyas*; 59% of total for *Calyptogena*), followed by MUFA (40% of total for *Maorithyas*; 27% of total for *Calyptogena*) (Figure 12). Among the individual fatty acids, the ubiquitous fatty acids C16:0 and C18:0 exhibited the highest abundance in both *Maorithyas* and *Calyptogena* sediments samples. These two fatty acids contributed 43% (*Maorithyas*) and 52% (*Calyptogena*) of the total fatty acids. The most abundant MUFA were $16{:}1^{\Delta 9}$ and $16{:}1^{\Delta 11}$. The only 10MBFA detected was 10Me-C16:0 (0.14% of total for *Maorithyas*; 1.37% of the total for *Calyptogena*). The TBFA consisted of the *iso* and *anteiso* isomers of C14:0, C15:0, C16:0 and C17:0 fatty acids. The most abundant TBFA was aC15:0 fatty acid (3.8% of the total for *Maorithyas*; 4.3% of the total for *Calyptogena*). The C17:0*cy* was the only CYFA detected from *Maorithyas* and *Calyptogena* sediment samples.

Table 3. Phospholipid fatty acid (PLFA) composition (μg g^{-1} dry weight; percentage of total in parenthesis) isolated from sediments of *Maorithyas* community.

Peak	Compound	B-74	C018	Average
1	C12:0	0.01 (0.1)	0.14 (0.2)	0.07 (0.2)
2	*i*C14:0	0.01 (0.2)	0.19 (0.2)	0.10 (0.2)
3	C14:1	nd	0.18 (0.2)	0.09 (0.2)
4	C14:0	0.14 (2.0)	4.49 (4.9)	2.32 (4.7)
5	*i*C15:0	0.07 (1.0)	0.96 (1.1)	0.52 (1.0)
6	*a*C15:0	0.14 (2.0)	3.58 (3.9)	1.86 (3.8)
7	C15:0	0.03 (0.4)	1.06 (1.2)	0.54 (1.1)
8	*i*C16:0	0.02 (0.3)	0.19 (0.2)	0.11 (0.2)
9	C16:1$^{\Delta 9}$c	0.19 (2.6)	0.62 (0.7)	0.41 (0.8)
10	C16:1$^{\Delta 9}$t	1.47 (20.2)	26.93 (29.3)	14.20 (28.6)
11	C16:1$^{\Delta 11}$c	0.09 (1.3)	1.87 (2.0)	0.98 (2.0)
12	C16:1$^{\Delta 11}$t	0.41 (5.7)	3.86 (4.2)	2.14 (4.3)
13	C16:0	0.96 (13.2)	22.92 (24.9)	11.94 (24.1)
14	10Me-C16:0	0.14 (1.9)	nd	0.07 (0.1)
15	*i*C17:0	0.05 (0.8)	0.72 (0.8)	0.39 (0.8)
16	*a*C17:0	0.05 (0.7)	0.35 (0.4)	0.20 (0.4)
17	C17:0*cy*	nd	2.26 (2.5)	1.13 (2.3)
18	C17:1$^{\Delta 9}$	0.13 (1.6)	nd	0.07 (0.1)
19	C17:0	0.05 (0.6)	1.04 (1.1)	0.54 (1.1)
20	C18:2	0.27 (3.7)	nd	0.13 (0.3)
21	C18:1$^{\Delta 9}$	0.45 (6.2)	0.68 (0.7)	0.56 (1.1)
22	C18:1$^{\Delta 11}$c	1.41 (19.5)	0.90 (1.0)	1.16 (2.3)
23	C18:1$^{\Delta 11}$t	nd	0.13 (0.1)	0.07 (0.1)
24	C18:0	0.57 (7.8)	18.37 (20.0)	9.47 (19.1)
25	C20:5	0.24 (3.4)	0.18 (0.2)	0.21 (0.4)
26	C20:1	0.17 (2.4)	0.18 (0.2)	0.17 (0.4)
27	C20:0	0.11 (1.5)	0.08 (0.1)	0.09 (0.2)
28	*C22:6*	*0.06 (0.8)*	*nd*	*0.03 (0.1)*

* nd - not detected

Table 4. Phospholipid fatty acid (PLFA) composition (μg g^{-1} dry weight; percentage of total in parenthesis) isolated from sediments of *Calyptogena* community.

Peak	Compound	374-2	C022	Average
1	C12:0	nd	nd	nd
2	*i*C14:0	0.14 (0.4)	0.06 (0.3)	0.10 (0.3)
3	C14:1	0.13 (0.3)	nd	0.07 (0.2)
4	C14:0	1.71 (4.3)	0.96 (4.4)	1.33 (4.3)
5	*i*C15:0	0.61 (1.5)	0.30 (1.4)	0.46 (1.5)
6	*a*C15:0	2.12 (5.3)	0.54 (2.5)	1.33 (4.3)
7	C15:0	0.59 (1.5)	0.26 (1.2)	0.42 (1.4)
8	*i*C16:0	0.16 (0.4)	0.13 (0.6)	0.15 (0.5)
9	C16:1$^{\Delta 9}$c	nd	nd	nd
10	C16:1$^{\Delta 9}$t	9.04 (22.5)	2.39 (11.1)	5.71 (18.5)
11	C16:1$^{\Delta 11}$c	0.87 (2.2)	0.27 (1.3)	0.57 (1.8)
12	C16:1$^{\Delta 11}$t	2.70 (6.7)	0.53 (2.4)	1.61 (5.2)
13	C16:0	9.72 (24.1)	7.40 (34.3)	8.56 (27.7)
14	10Me-C16:0	0.35 (0.9)	0.50 (2.3)	0.42 (1.4)
15	*i*C17:0	0.28 (0.7)	0.52 (2.4)	0.40 (1.3)
16	*a*C17:0	0.32 (0.8)	0.62 (2.9)	0.47 (1.5)
17	C17:0*cy*	1.14 (2.8)	0.79 (3.7)	0.97 (3.1)
18	C17:1$^{\Delta 9}$	nd	nd	nd
19	C17:0	0.57 (1.4)	0.26 (1.2)	0.42 (1.3)
20	C18:2	nd	nd	nd
21	C18:1$^{\Delta 9}$	0.29 (0.7)	0.11 (0.5)	0.20 (0.6)
22	C18:1$^{\Delta 11c}$	0.30 (0.8)	0.13 (0.6)	0.22 (0.7)
23	C18:1$^{\Delta 11}$t	nd	nd	nd
24	C18:0	9.09 (22.6)	5.83 (27.0)	7.46 (24.1)
25	C20:5	nd	nd	nd
26	C20:1	0.06 (0.1)	nd	0.03 (0.1)
27	C20:0	0.05 (0.1)	nd	0.03 (0.1)
28	C22:6	nd	nd	nd

* nd – not detected

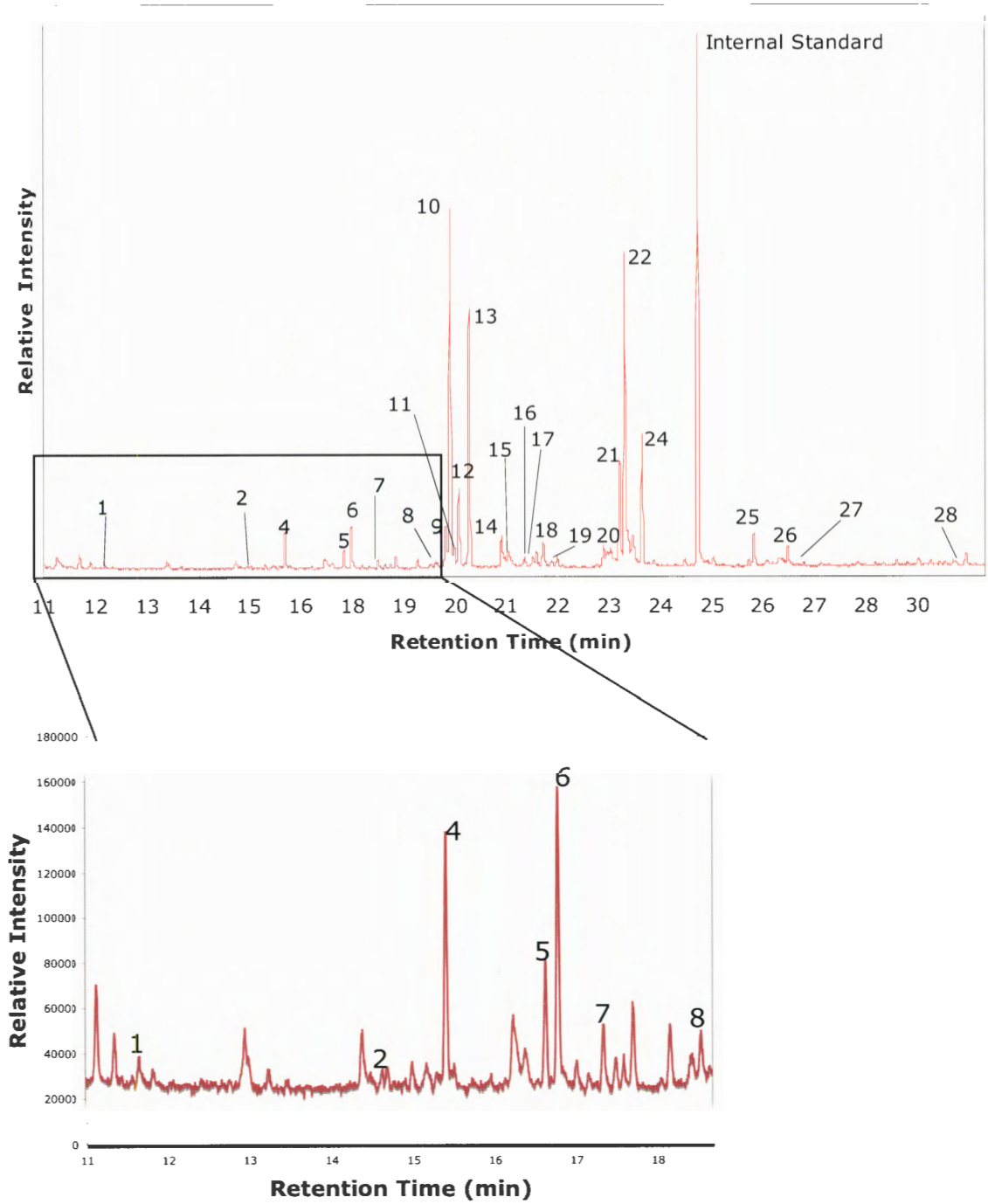

Figure 7. Total ion chromatogram of phospholipid fatty acids isolated from B-74, *Maorithyas* sediments.

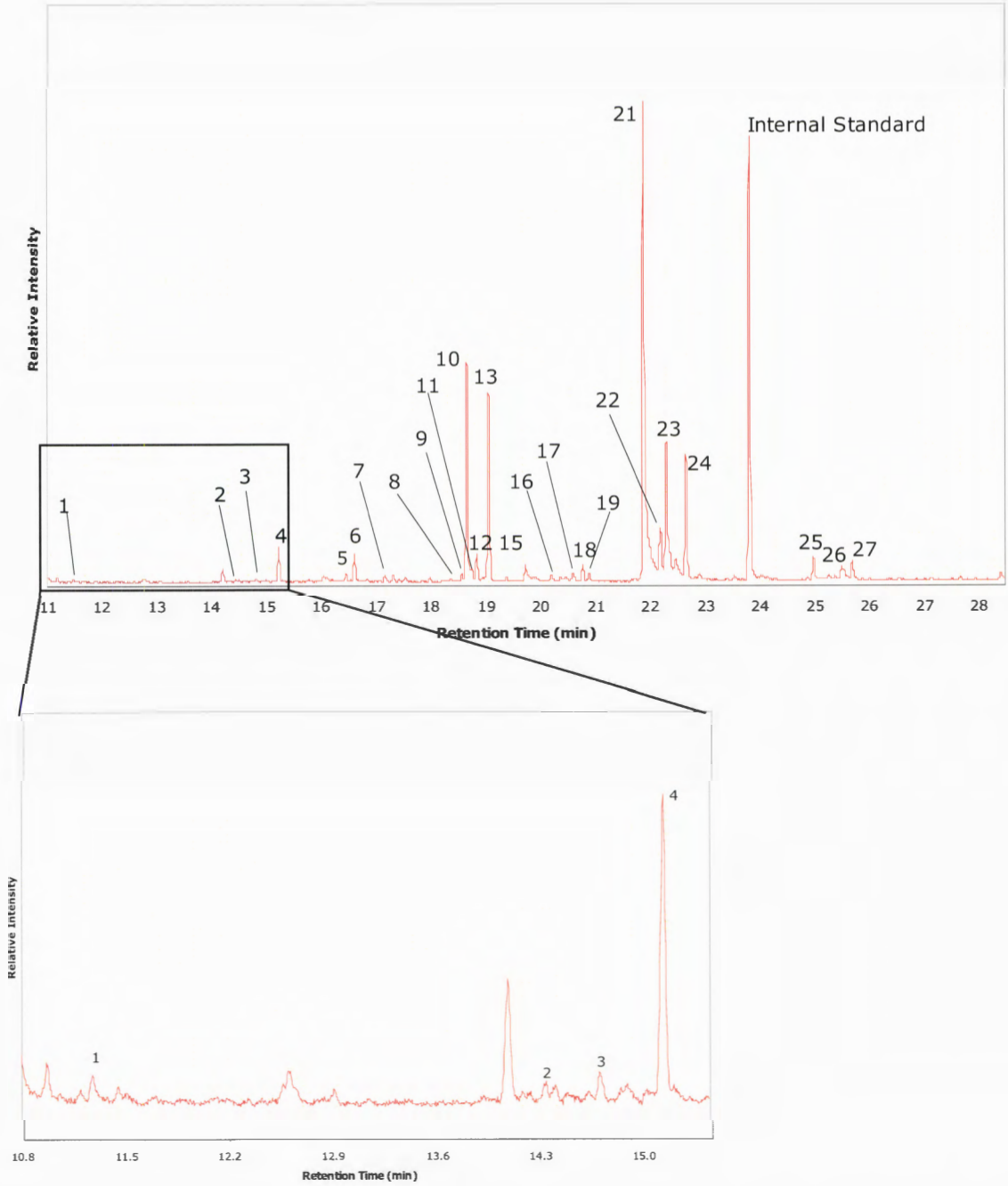

Figure 8. Total ion chromatograms of phospholipid fatty acids isolated from C018, *Maorithyas* sediments.

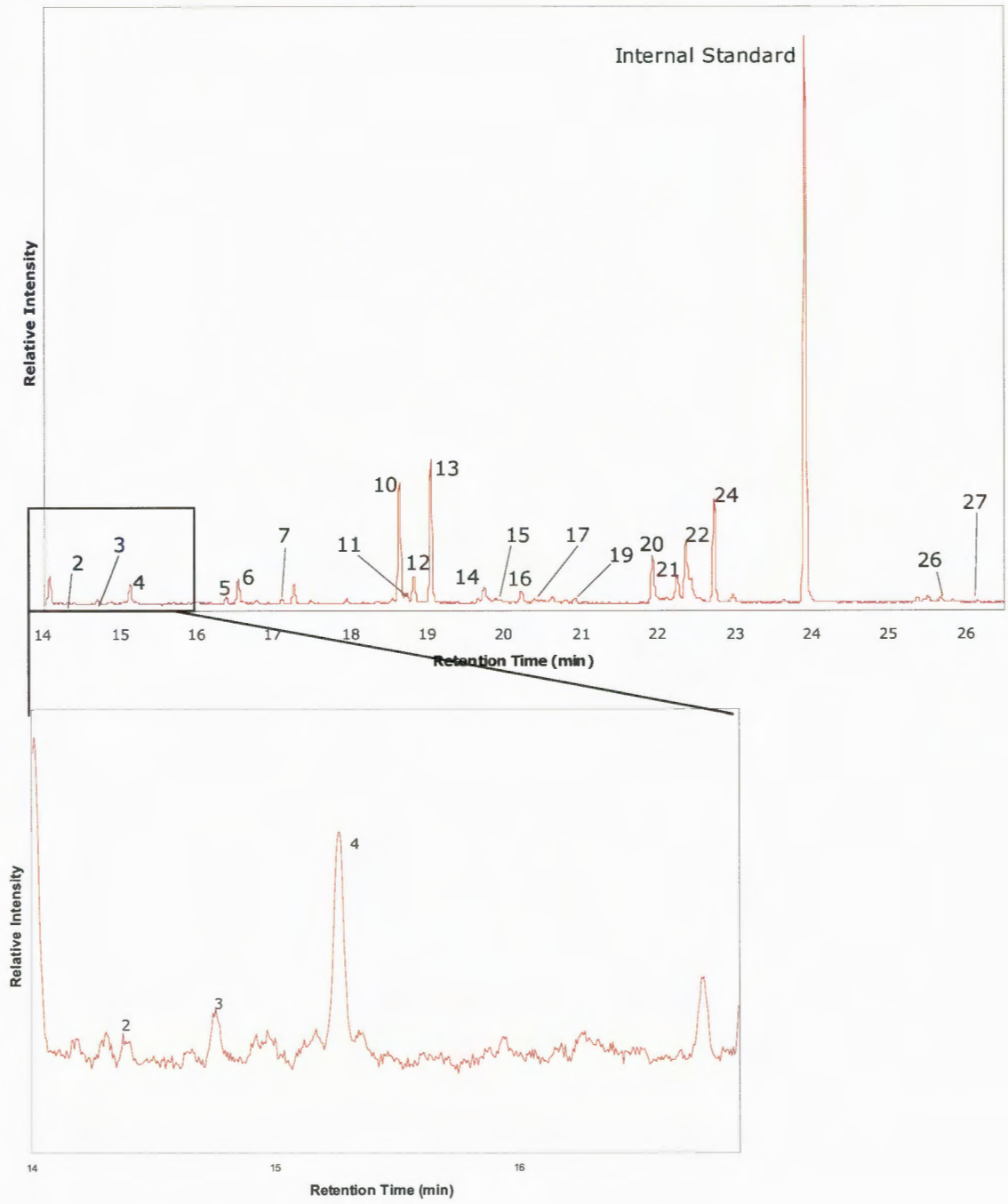

Figure 9. Total ion chromatograms of phospholipid fatty acids isolated from 374-2, *Calyptogena* sediments.

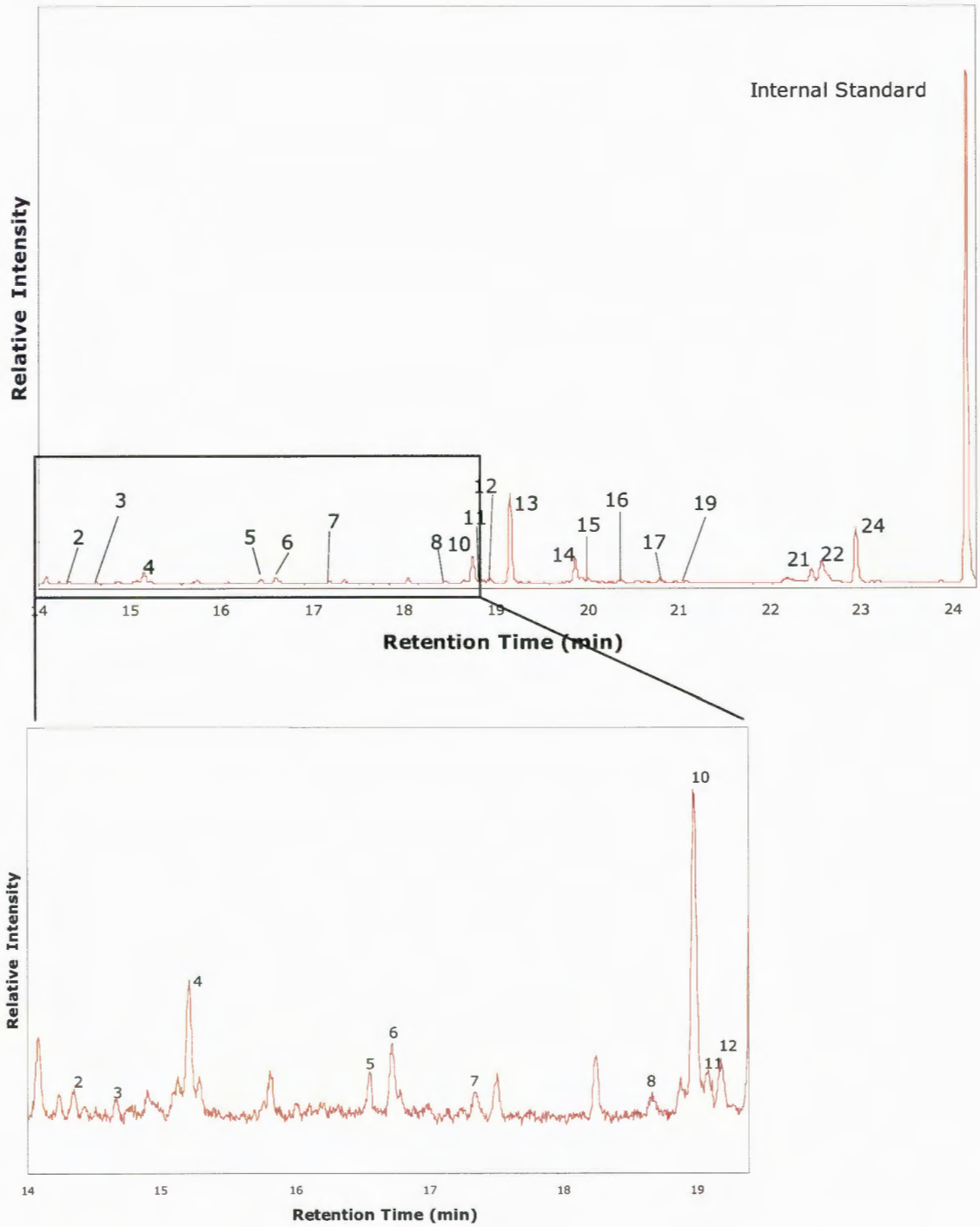

Figure 10. Total ion chromatograms of phospholipid fatty acids isolated from C022, *Calyptogena* sediments.

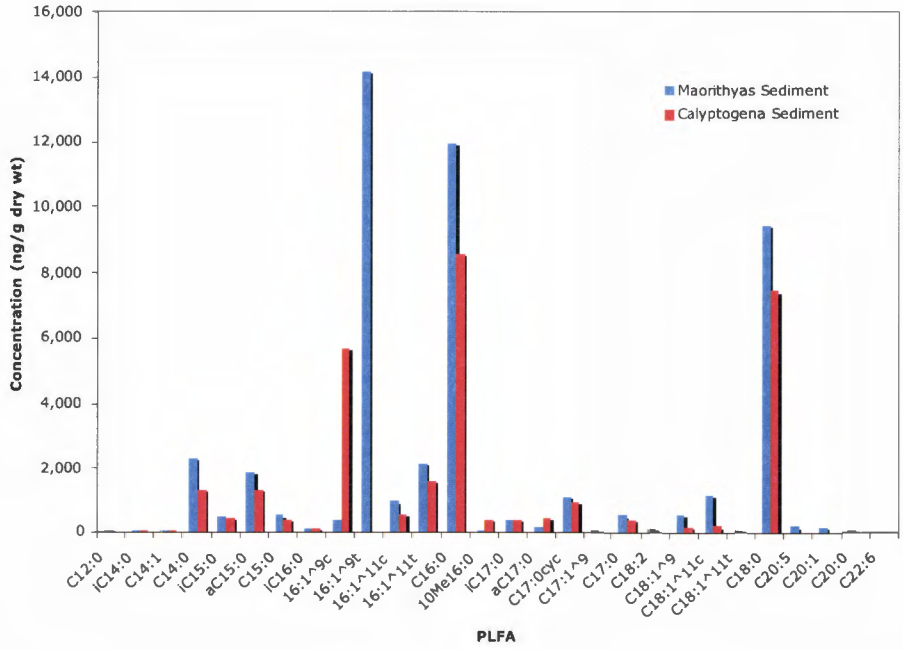

Figure 11. Distribution of PLFA in *Maorithyas* and *Calyptogena* sediments.

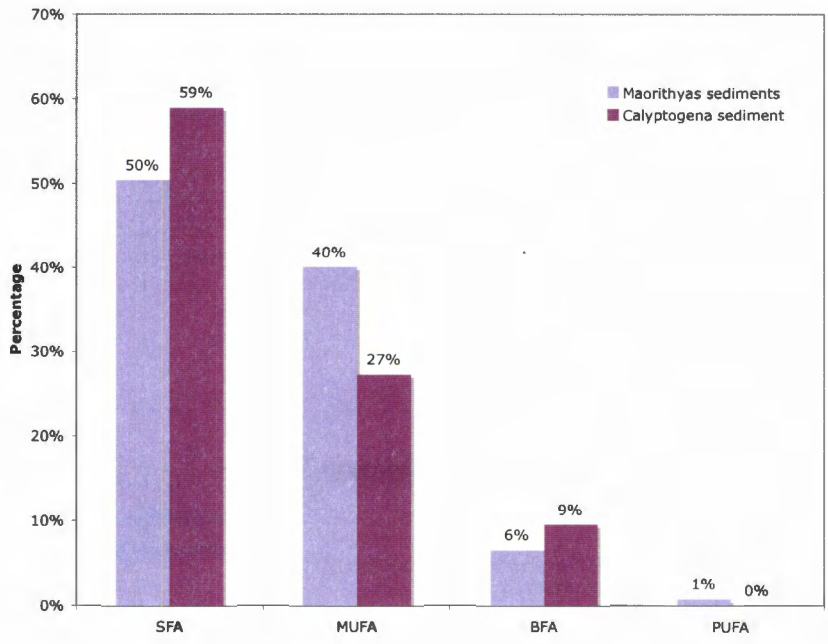

Figure 12. Relative contributions of different categories of fatty acid from the *Maorithyas* and *Calyptogena* sediments.

3.1.2. PLFA Profiles of Clam Tissues

The PLFA profiles for the *Maorithyas hadalis* tissue samples are shown in Table 5 and Figure 13. A total of 24 and 26 fatty acids were detected from leg and gill tissue, respectively. They include SAFA, MUFA, PUFA, CYFA, and TBFA. The lipid profiles were characterized by large proportions of MUFA, (93.5% of total for gill; 75.9% of total for leg) with C16:$1^{\Delta 9}$ and C18:$1^{\Delta 9}$ predominating. SAFA were less abundant and accounted for 5.8% of gill sample and 16.2% of leg sample. Several PUFA were present in both samples, including C18:2, C20:4, C20:5 isomers and C22:2. TBFA (*i*C15:0 and *i*C17:0) were found in very low concentrations in samples as well as CYFA (C19:0*cy*).

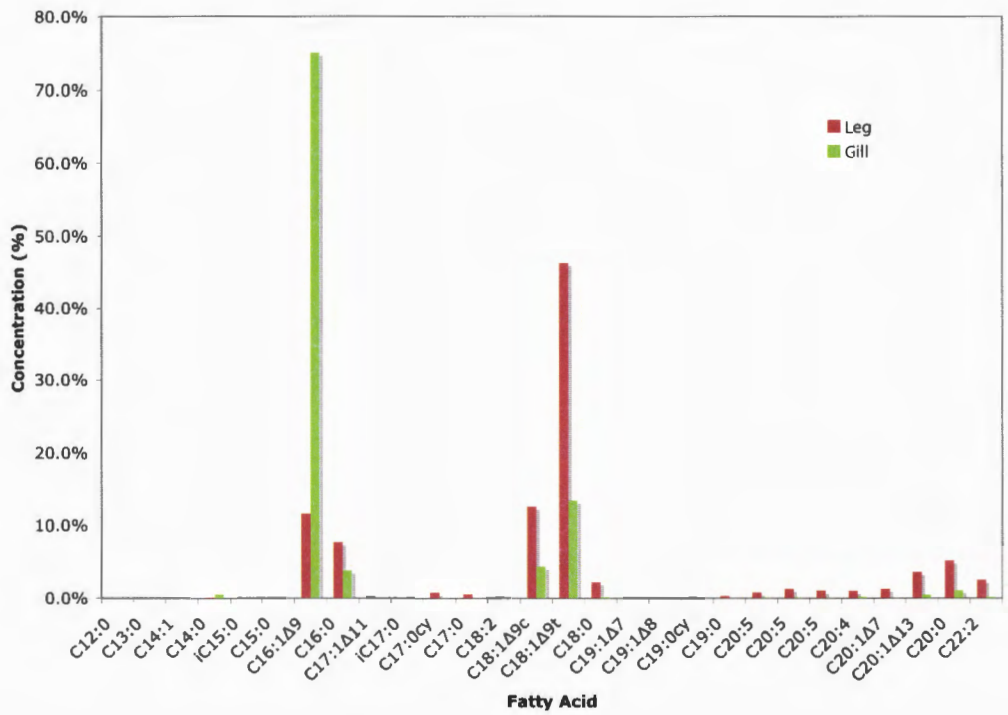

Figure 13. Comparison of the phospholipid fatty acid compositions of *Maorithyas hadalis* gill and leg tissues.

Table 5. Phospholipid fatty acid (PLFA) composition (mg g^{-1} dry weight; percentage of total in parenthesis) of tissue samples isolated from *Maorithyas hadalis*.

Compound	Leg	Gill
C12:0	12.60 (0.1)	0.25 (0.0)
C13:0	nd	0.10 (0.0)
C14:1	nd	1.52 (0.1)
C14:0	27.00 (0.1)	17.67 (0.6)
*i*C15:0	nd	0.36 (0.0)
C15:0	17.10 (0.1)	2.74 (0.1)
C16:1$^{\Delta 9}$	2564.10 (11.7)	2365.96 (75.0)
C16:0	1692.00 (7.7)	120.14 (3.8)
C17:1$^{\Delta 11}$	58.88 (0.3)	nd
*i*C17:0	11.70 (0.1)	0.33 (0.0)
C17:0*cy*	171.90 (0.8)	1.95 (0.1)
C17:0	129.60 (0.6)	1.58 (0.1)
C18:2	36.34 (0.2)	0.54 (0.0)
C18:1$^{\Delta 9}$c	2782.80 (12.6)	136.68 (4.3)
C18:1$^{\Delta 9}$t	10220.40 (46.3)	424.08 (13.5)
C18:0	488.70 (2.2)	5.77 (0.2)
C19:1$^{\Delta 7}$	35.51 (0.2)	0.40 (0.0)
C19:1$^{\Delta 8}$	nd	1.18 (0.0)
C19:0*cy*	42.30 (0.2)	0.63 (0.0)
C19:0	71.10 (0.3)	nd
C20:5	173.75 (0.8)	2.56 (0.1)
C20:5	285.98 (1.3)	2.28 (0.1)
C20:5	239.19 (1.1)	1.20 (0.0)
C20:4	223.71 (1.0)	6.74 (0.2)
C20:1$^{\Delta 7}$	281.60 (1.3)	1.61 (0.1)
C20:1$^{\Delta 13}$	796.37 (3.6)	15.79 (0.5)
C20:0	1138.50 (5.2)	35.29 (1.1)
C22:2	560.25 (2.6)	5.76 (0.2)

3.2. Hopanoid Profiles

3.2.1. Hopanoid Profiles of Sediment Samples

The hopanoids profiles of two communities are shown in Tables 6 and 7. The mass spectra of (22R)-17β(H),21β(H)-homopan-31-ol and (22R)-17β(H),21β(H)-bishomopan-32-ol are shown in Figures 14 and 15. A total of 5 hopanoids were detected from the sediment samples, including C_{29}17α(H),21β(H)-norhopane, C_{30}17α(H),21β(H)-norhopane, hopan-29-ol, (22R)-17β (H),21β(H)-homopan-31-ol and (22R)-17β(H),21β (H)-bishomopan-32-ol. Hopan-29-ol was the only hopanoid not present in sample 374-2 from the *Calyptogena* community sediment. C_{30}17α (H),21β(H)-norhopane was the most abundant hopanoid detected, followed by either (22R)-17β(H),21β (H)-bishomopan-32-ol (*Maorithyas* sediment) or C_{29}17α(H),21β(H)-norhopane (*Calyptogena* sediment).

Table 6. Concentration of hopanoids (μg g^{-1} dry weight; percentage in parenthesis) isolated from sediments of the *Maorithyas* community.

Compound	B-74	C018	Average
C_{29}17α(H),21β(H)-norhopane	0.90 (4.2)	0.59 (4.4)	0.74 (4.3)
C_{30}17α(H),21β(H)-norhopane	8.69 (40.4)	2.86 (21.4)	5.77 (33.1)
hopan-29-ol	3.71 (17.2)	2.34 (17.5)	3.03 (17.4)
(22R)-17β(H),21β(H)-homopan-31-ol	3.21 (14.9)	3.14 (23.5)	3.18 (18.2)
(22R)-17β(H),21β(H)-bishomopan-32-ol	5.01 (23.3)	4.43 (33.2)	4.72 (27.1)

Table 7. Concentration of hopanoids (μg g^{-1} dry weight; percentage in parenthesis) isolated from sediments of the *Calyptogena* community.

Compound	374-2	C022	Average
C_{29}17α(H),21β(H)-norhopane	0.85 (21.8)	2.61(17.5)	1.73 (18.4)
C_{30}17α(H),21β(H)-norhopane	0.53 (13.5)	8.35 (56.0)	4.44 (47.2)
hopan-29-ol	nd	1.67 (11.2)	0.84 (8.9)
(22R)-17β(H),21β(H)-homopan-31-ol	0.54 (13.7)	1.27 (8.5)	0.91 (9.6)
(22R)-17β(H),21β(H)-bishomopan-32-ol	1.99 (50.9)	1.00 (6.7)	1.50 (15.9)

*nd – not detected

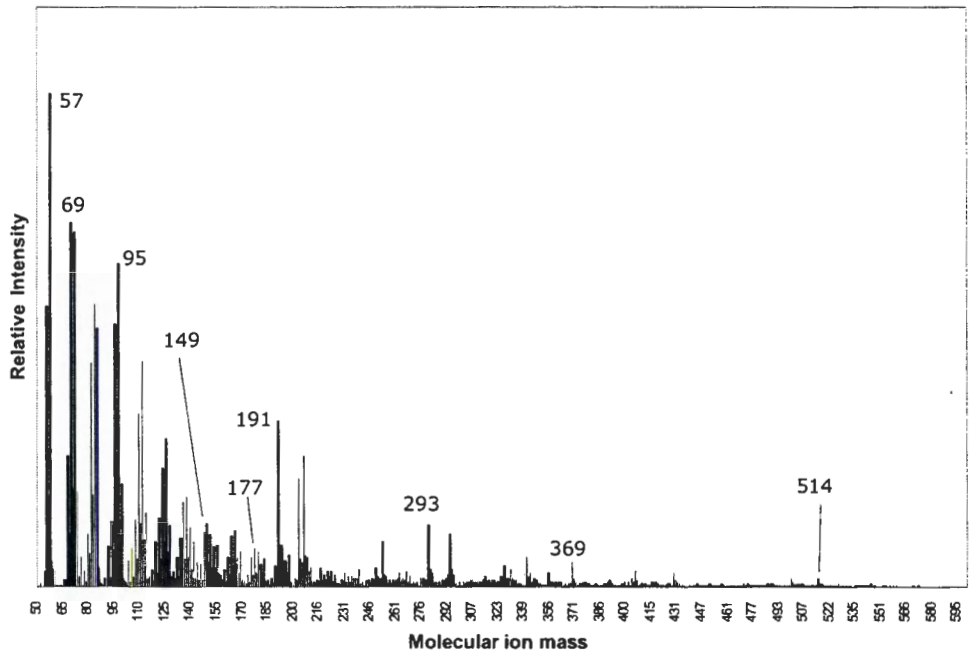

Figure 14. Mass spectrum of hopanoid, (22R)-17β(H),21β(H)-homopan-31-ol.

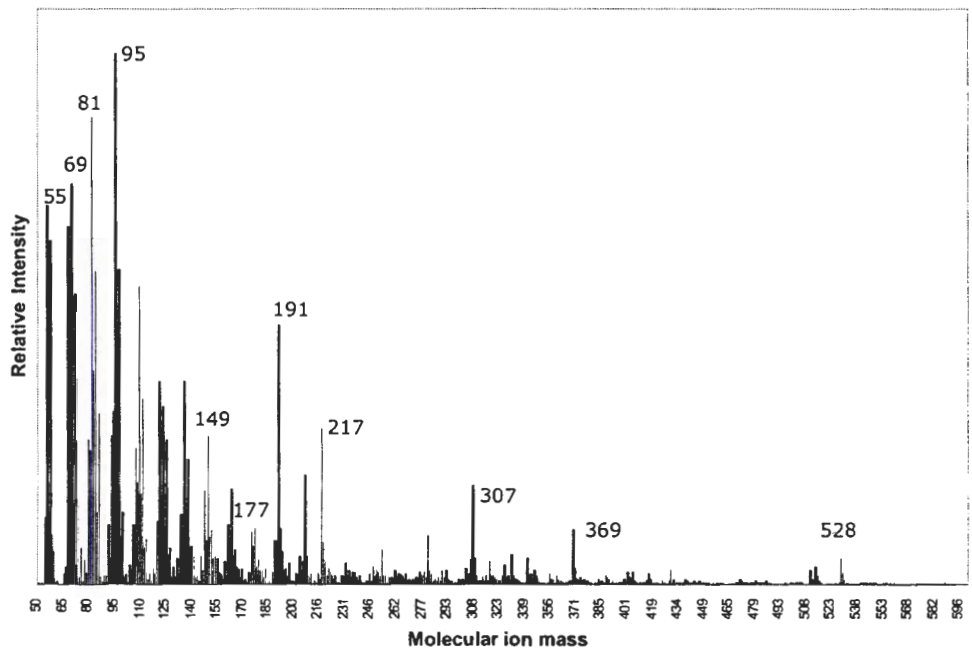

Figure 15. Mass spectrum of hopanoid, (22R)-17β(H),21β(H)-bishomopan-32-ol.

3.2.2. Hopanoids Profiles of Clam Tissues

Hopanoid biomarkers were not detected from the *Maorithyas hadalis* organ samples.

3.3. Glycolipid Fatty Acid (GLFA) Profiles

3.3.1. GLFA Profiles of Sediment Samples

GLFA profiles of the *Maorithyas* and *Calyptogena* sediment samples are listed in Tables 8 and 9. Glycolipid fatty acids with chain length of 11 to 20 carbons were detected from the sediments. These GLFA include SAFA, MUFA, CYFA, and TBFA. The GLFA profiles are predominated by SAFA (61% of the total for *Maorithyas*; 80% of the total for *Calyptogena*). All the major fatty acids in GLFA profiles were also found in PLFA profiles. The ubiquitous fatty acids C16:0 and C18:0 were the most abundant fatty acids among the lipid profiles. The MUFA was less abundant, accounting 33% and 15% for *Maorithyas* and *Calyptogena* sediment, respectively. The most abundant MUFA was C16:1$^{\Delta 9}$. *Cis* and *tran* C18:1$^{\Delta 9}$ were also detected. Several TBFA, such as *a*C15:0, *i*C16:0 and *i*C17:0, and one cyclopropane, C17:0*cy* were also present in the samples.

Table 8, Concentration of glycolipid fatty acids (GLFA) (μg g^{-1} dry weight; percentage of total in parenthesis) isolated from sediment of *Maorithyas* community.

Compound	B-74	C-018	Average
C11:0	0.03 (0.0)	nd	0.01 (0.0)
C12:0	1.69 (1.4)	1.88 (0.8)	1.79 (1.0)
C13:0	0.06 (0.1)	0.11 (0.0)	0.09 (0.1)
C14:0	4.01 (3.4)	7.11 (2.9)	5.56 (3.0)
*a*C15:0	5.34 (4.5)	9.62 (3.9)	7.48 (4.1)
C15:0	0.24 (0.2)	1.18 (0.5)	0.71 (0.4)
C16:$1^{\Delta 9}$	17.43 (14.8)	59.15 (23.8)	38.29 (20.9)
C16:0	30.37 (25.8)	47.26 (19.0)	38.81 (21.2)
*i*C17:0	0.38 (0.3)	0.29 (0.1)	0.33 (0.2)
C17:0cy	0.61 (0.5)	5.14 (2.1)	2.87 (1.6)
C17:0	0.56 (0.5)	0.76 (0.3)	0.66 (0.4)
C18:$1^{\Delta 9}$c	5.35 (4.6)	4.78 (1.9)	5.06 (2.8)
C18:$1^{\Delta 9}$t	10.44 (8.9)	24.56 (9.9)	17.50 (9.6)
C18:0	39.33 (33.4)	84.59 (34.0)	61.96 (33.8)
C19:0	0.20 (0.2)	0.28 (0.1)	0.24 (0.1)
C20:0	1.73 (1.5)	1.99 (0.8)	1.86 (1.0)

*nd – not detected

Table 9, Concentration of glycolipid fatty acids (GLFA) (μg g^{-1} dry weight; percentage of total in parenthesis) isolated from sediment of *Calyptogena* community.

Compound	374-2	C-022	Average
C12:0	0.84 (0.6)	1.34 (1.8)	1.09 (1.0)
C14:0	3.17 (2.3)	0.98 (1.3)	2.07 (1.9)
*a*C15:0	3.74 (2.7)	0.61 (0.8)	2.17 (2.0)
C15:0	0.52 (0.4)	0.22 (0.3)	0.37 (0.4)
*i*C16:0	nd	0.66 (0.8)	0.33 (0.3)
C16:$1^{\Delta 9}$	11.98 (8.7)	9.89 (13.2)	10.93 (10.2)
C16:0	31.24 (22.5)	13.41 (17.9)	22.32 (20.9)
*i*C17:0	0.86 (0.6)		0.43 (0.4)
C17:0cy	2.27 (1.6)	2.17 (2.9)	2.22 (2.1)
C17:0	0.67 (0.5)	0.36 (0.5)	0.52 (0.5)
C18:$1^{\Delta 9}$c	2.10 (1.5)	nd	1.05 (1.0)
C18:$1^{\Delta 9}$t	8.53 (6.1)	nd	4.26 (4.0)
C18:0	71.43 (51.4)	44.87 (59.9)	58.15 (54.4)
C20:0	1.63 (1.2)	0.46 (0.6)	1.04 (1.0)

* nd - not detected

3.3.2. GLFA Profiles of Clam Tissues

The concentrations of the major GLFA isolated from the *Maorithyas hadalis* tissue samples are listed in Table 10. Fatty acids with 12 to 22 carbons were detected from the leg and gill samples, including SAFA, MUFA, and TBFA. PUFA (C18:2 and C22:2) were only detected from the gill sample, constituting 7% of the total fatty acids. For the leg sample, its GLFA profile was dominated by SAFA (65%), followed by MUFA (33%). The most abundant fatty acids were C16:0 and C18:0. For the gill tissue, the concentration of MUFA (82%) was higher than the concentration of SAFA (11%). The C16:1$^{\Delta 9}$ and C18:1$^{\Delta 9}$t were the major fatty acids found in the gill sample. Several TBFA (iC15:0, aC15:0 iC17:0) were also present in both leg (2.3%) and gill (0.1%) samples, but in very low concentration.

Table 10, Concentration of glycolipid fatty acids (GLFA) (mg g^{-1} dry weight; percentage in parenthesis) isolated from *Maorithyas hadalis* organ samples.

Compound	Leg	Gill
C12:0	0.21 (0.8)	0.01 (0.1)
C14:1	nd	0.03 (0.5)
C14:0	0.30 (1.1)	0.02 (0.3)
*i*C15:0	0.03 (0.1)	nd
*a*C15:0	0.03 (0.1)	nd
C15:0	0.06 (0.2)	0.01 (0.2)
C16:1$^{\Delta 9}$	1.32 (4.8)	1.24 (20.8)
C16:0	3.03 (11.0)	0.22 (3.7)
*i*C17:0	0.57 (2.1)	0.01 (0.1)
C17:0	0.12 (0.4)	0.01 (0.3)
C18:2$^{\Delta 9,12}$	nd	0.28 (4.6)
C18:1$^{\Delta 9}$c	2.43 (8.8)	0.33 (5.6)
C18:1$^{\Delta 9}$t	5.37 (19.4)	3.34 (55.7)
C18:0	12.39 (44.8)	0.08 (1.4)
C19:0	0.12 (0.4)	nd
C20:0	1.68 (6.1)	0.29 (4.8)
C22:2	nd	0.12 (2.0)

* nd – not detected

3.4. Sterol Profiles

3.4.1. Sterol Profiles of Sediment Samples

Sterol profiles of sediment samples are shown in Tables 11 and 12. The relative distribution of C_{28}, C_{29}, C_{30} and C_{31} sterols are shown in Figure 16 and the mass spectra of selected sterols are shown in Figures 17 and 18. A number of methyl- and ethyl-sterols were detected in both community sediment samples. Both communities were dominated by either 24-ethylcholest-5-en-3β-ol (*Maorithyas* 37.5%) or cholest-5-en-3β-ol (*Calyptogena* 34.3%). The higher plants sterol biomarkers were found in the profiles; e.g., 24-ethylcholesta-5,22-dien-3β-ol, 24-ethyl-5α-cholest-22-en-3β-ol, 24-ethylcholest-5-en-3β-ol and 24-ethyl-5α-cholestan-3β-ol. Some sterol biomarkers of algae were also detected in the cold seep sediment, such as 24-methylcholesta-5,22-dien-3β-ol, cholesta-5,22(E)-dien-3β-ol, cholest-5-en-3β-ol.

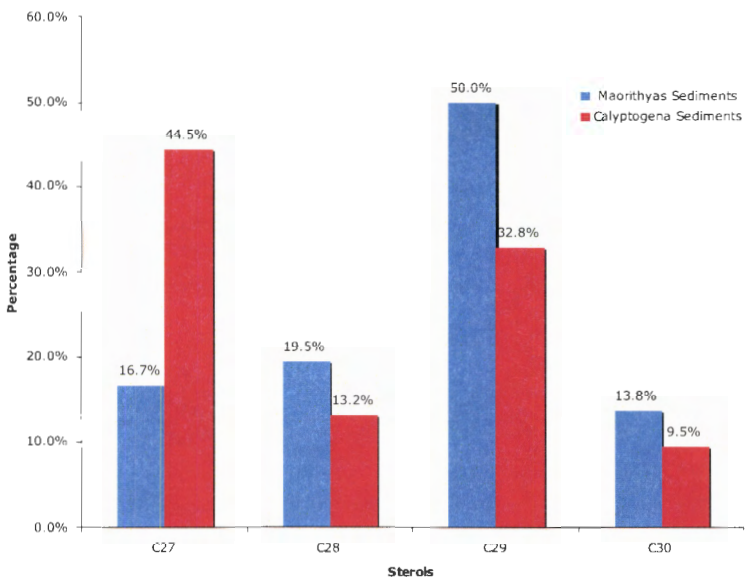

Figure 16. Relative distribution of sterols isolated from *Maorithyas* and *Calyptogena* sediments.

Table 11. Concentration of sterols (μg g^{-1} dry weight; percentage of total in parenthesis) isolated from the *Maorithyas* sediments.

Compound	B74	C018	Average
24-norcholesta-5,22 dien-3ß-ol	nd	2.14 (2.0)	1.07 (0.8)
24-nor-5α-cholest-22-en-3β-ol	nd	0.94 (0.9)	0.47 (0.3)
C_{27} $^{\Delta 5,22}$(Z)	3.55 (2.1)	nd	1.78 (1.3)
24-nor-24-methylcholesta-5,22-dien-3β-ol	2.96 (1.8)	nd	1.48 (1.1)
Cholesta-5,22(E)-dien-3β-ol	nd	4.08 (3.8)	2.04 (1.5)
5α-cholest-22(E)-en-3β-ol	2.39 (1.4)	2.19 (2.1)	2.29 (1.7)
Cholest-5-en-3β-ol	10.88 (6.5)	12.49 (11.8)	11.69 (8.5)
5α-cholestan-3β-ol	2.03 (1.2)	2.06 (1.9)	2.04 (1.5)
24-methylcholesta-5,22-dien-3β-ol	7.31 (4.4)	10.18 (9.6)	8.75 (6.4)
24-methyl-5α-cholest-22-en-3-ol	1.29 (0.8)	2.19 (2.1)	1.74 (1.3)
24-methylenecholest-5-en-3β-ol	3.37 (2.0)	8.87 (8.3)	6.12 (4.5)
24-methylcholest-5-en-3β-ol	6.22 (3.7)	12.15 (11.4)	9.19 (6.7)
24-methyl-5α-cholestan-3β-ol	1.70 (1.0)	nd	0.85 (0.6)
23,24-dimethylcholesta-5,22-dien-3β-ol	2.31 (1.4)	1.68 (1.6)	1.99 (1.5)
24-ethylcholesta-5,22-dien-3β-ol	6.42 (3.8)	6.21 (5.8)	6.32 (4.6)
24-ethyl-5α-cholest-22-en-3β-ol	2.17 (1.3)	2.10 (2.0)	2.13 (1.6)
24-ethylcholest-5-en-3β-ol	90.73 (54.3)	11.85 (11.2)	51.29 (37.5)
24-ethyl-5α-cholestan-3β-ol	5.33 (3.2)	nd	2.66 (1.9)
24(Z)ethylidenecholest-5-en-3β-ol	nd	7.80 (7.3)	3.90 (2.9)
4α,23,24-trimethylcholest-22-en-3β-ol	10.00 (6.0)	9.10 (8.6)	9.55 (7.0)
24-ethylcholest-7-en-3β-ol	3.07 (1.8)	4.16 (3.9)	3.61 (2.6)
24(Z)propylidenecholest-5-en-3β-ol	1.48 (0.9)	1.59 (1.5)	1.54 (1.1)
4,23,24-trimethyl-5α-cholestan-3β-ol	3.97 (2.4)	4.49 (4.2)	4.23 (3.1)

* nd – not detected

Table 12. Concentration of sterols (μg g^{-1} dry weight; percentage of total in parenthesis) isolated from *Calyptogena* sediments.

Compound	374-2	C022	Average
24-norcholesta-5,22 dien-3ß-ol	0.32 (0.7)	nd	0.16 (0.5)
24-nor-5α-cholest-22-en-3β-ol	0.45 (1.0)	nd	0.23 (0.6)
Cholesta-5,22(E)-dien-3β-ol	1.94 (4.5)	nd	0.97 (2.8)
5α-cholest-22(e)-en-3β-ol	1.95 (4.5)	nd	0.98 (2.8)
Cholest-5-en-3β-ol	5.49 (12.6)	18.42(69.8)	11.96(34.3)
5α-cholestan-3β-ol	2.46 (5.7)	nd	1.23 (3.5)
24-methylcholesta-5,22-dien-3β-ol	3.83 (8.8)	nd	1.91 (5.5)
24-methyl-5α-cholest-22-en-3-ol	1.83 (4.2)	nd	0.91 (2.6)
24-methylcholest-5-en-3β-ol	1.92 (4.4)	nd	0.96 (2.8)
24-methyl-5α-cholestan-3β-ol	1.63 (3.8)	nd	0.82 (2.3)
23,24-dimethylcholesta-5,22-dien-3β-ol	1.11 (2.6)	nd	0.55 (1.6)
23,24-dimethylcholesta-5,22-dien-3β-ol	3.92 (9.0)	nd	1.96 (5.6)
24-ethyl-5α-cholest-22-en-3β-ol	1.37 (3.2)	nd	0.69 (2.0)
24-ethylcholest-5-en-3β-ol	4.66 (10.7)	7.99 (30.2)	6.33 (18.1)
24-ethyl-5α-cholestan-3β-ol	3.87 (8.9)	nd	1.94 (5.6)
4-methyl,24-ethylcholest-22-en-3β-ol	0.64 (1.5)	nd	0.32 (0.9)
24-proplyl-5α-cholestan-3β-ol	1.83 (4.2)	nd	0.92 (2.6)
C_{30} 4-methylstanol	4.15 (9.6)	nd	2.08 (5.9)

* nd – not detected

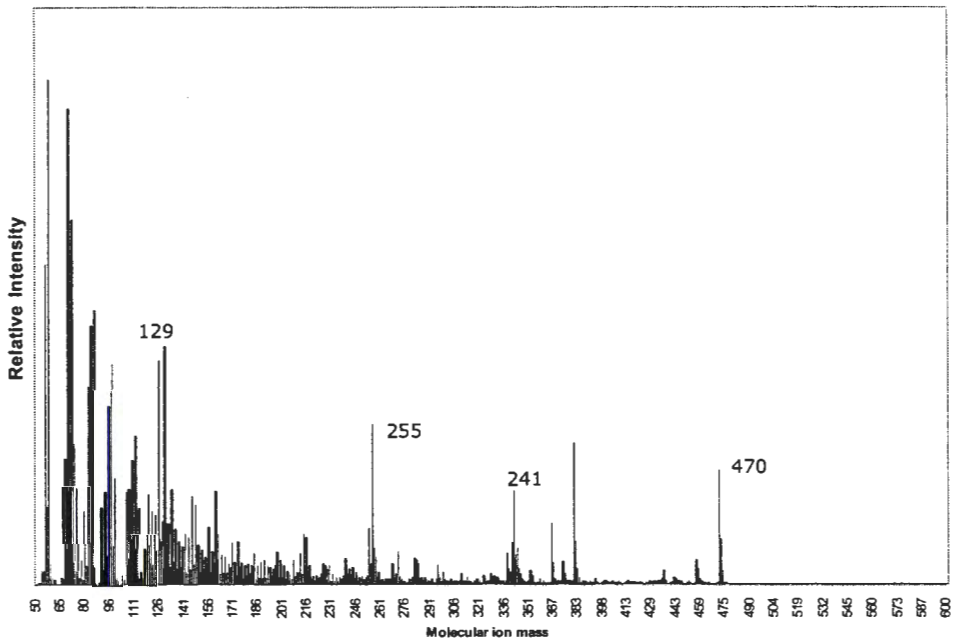

Figure 17. Mass spectrum of sterol, 24-methylcholesta-5,22-dien-3β-ol.

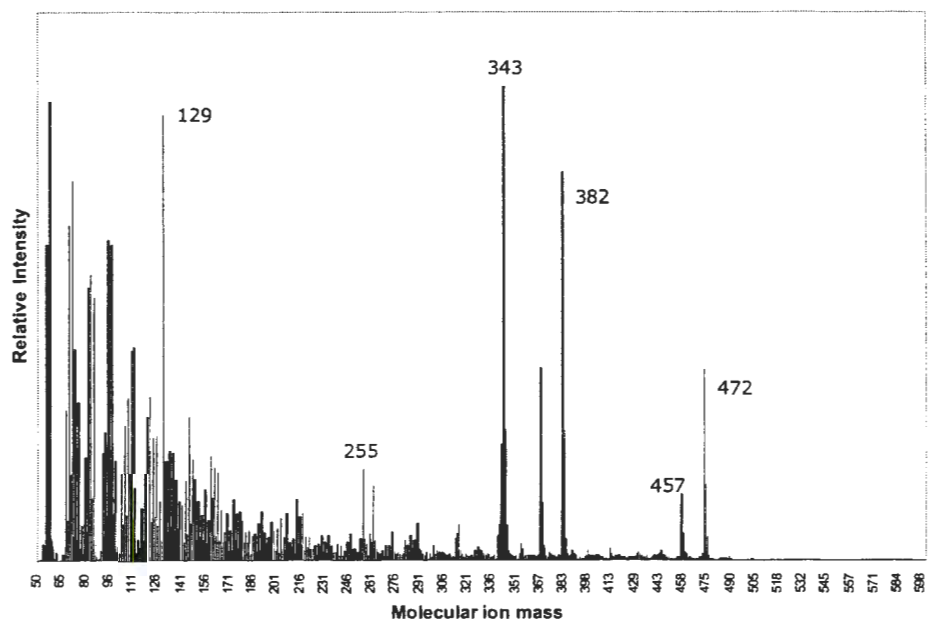

Figure 18. Mass spectrum of sterol, 24-methylcholest-5-en-3β-ol.

3.4.2. Sterols Profiles of Clam Tissues

Only six sterols were detected in the *Maorithyas hadalis* leg tissue (Table 13). These sterols include cholest-5-en-3β-ol, 5α-cholestan-3β-ol, C_{28} $^{\Delta5,22}$ sterol, 24-methylenecholest-5-en-3β-ol, 24-methylcholest-5-en-3β-ol and 24-ethylcholest-5-en-3β-ol. Cholest-5-en-3β-ol was the only sterol isolated from the *Maorithyas hadalis* gill tissue.

Table 13. Concentration of sterols (mg g^{-1} dry weight; percentage of total in parenthesis) isolated from the *Maorithyas hadalis* tissue samples.

Compound	Leg	Gill
Cholest-5-en-3β-ol	3.76 (45.2)	0.01 (100)
5α-cholestan-3β-ol	1.14 (13.7)	
C_{28} Δ5,22 sterol	1.51 (18.1)	
24-methylenecholest-5-en-3β-ol	0.56 (6.7)	
24-methylcholest-5-en-3β-ol	0.54 (6.4)	
24-ethylcholest-5-en-3β-ol	0.82 (9.9)	

3.5. *n*-Alkane Profiles

3.5.1. *n*-Alkanes of Sediment Samples

The concentrations of individual *n*-alkanes in sediments are shown in Tables 14 and 15. Long chain *n*-alkanes, ranging from 21 to 31 carbons were detected from two community sediment samples. The *n*-alkanes were dominated by odd numbered C_{27}, C_{29} and C_{31} which accounted for 52% and 40% of total *n*-alkanes for the *Maorithyas* and *Calyptogena* sediments, respectively.

Table 14. Concentration of *n*-alkanes (μg g^{-1} dry weight; percentage of total in parenthesis) isolated from the *Maorithyas* sediment.

Compound	B-74	C018	Average
C_{21}	0.77 (1.8)	0.61 (2.8)	0.69 (2.2)
C_{22}	0.76 (1.8)	1.11 (5.2)	0.94 (2.9)
C_{23}	0.63 (1.5)	0.66 (3.0)	0.64 (2.0)
C_{24}	1.59 (3.8)	1.59 (7.4)	1.59 (5.0)
C_{25}	1.73 (4.1)	1.02 (4.7)	1.38 (4.3)
C_{26}	1.64 (3.9)	1.16 (5.4)	1.40 (4.4)
C_{27}	3.33 (7.9)	2.19 (10.1)	2.76 (8.7)
C_{28}	3.66 (8.7)	1.89 (8.8)	2.78 (8.7)
C_{29}	8.15 (19.4)	3.78 (17.5)	5.96 (18.8)
C_{30}	8.54 (20.4)	3.32 (15.4)	5.93 (18.7)
C_{31}	11.16 (26.6)	4.26 (19.7)	7.71 (24.3)
$C_{27,29,31}$	22.64 (53.9)	10.23 (47.3)	16.43 (51.8)

Table 15. Concentration of *n*-alkanes (μg g^{-1} dry weight; percentage of total in parenthesis) isolated from the *Calyptogena* sediment.

Compound	374-2	C022	Average
C_{21}	0.34 (3.1)	0.41 (0.6)	0.38 (1.0)
C_{22}	0.80 (7.3)	1.20 (1.8)	1.00 (2.6)
C_{23}	0.45 (4.1)	0.64 (1.0)	0.54 (1.4)
C_{24}	0.93 (8.4)	16.23 (24.9)	8.58 (22.6)
C_{25}	0.71 (6.4)	1.76 (2.7)	1.23 (3.2)
C_{26}	0.81 (7.4)	2.30 (3.5)	1.56 (4.1)
C_{27}	1.01 (9.2)	3.97 (6.1)	2.49 (6.6)
C_{28}	0.96 (8.7)	8.78 (13.5)	4.87 (12.8)
C_{29}	1.60 (14.6)	9.16 (14.1)	5.38 (14.2)
C_{30}	1.47 (13.4)	7.83 (12.0)	4.65 (12.2)
C_{31}	1.92 (17.5)	12.76 (19.6)	7.34 (19.3)
$C_{27,29,31}$	4.53 (41.3)	25.89 (39.8)	15.21 (40.1)

3.5.2. *n*-Alkane Profiles of Clam Tissues

No *n*-alkane was detected from the organ samples, but high concentrations of sulfur were detected in the samples.

3.6. Microbial Biomass Estimation

The use of PLFA for estimating viable biomass relies on a conversion factor from the mass of PLFA to the mass of microbes (Green and Scow, 1999). The conversion factor described by Balkwill et al. (1988) was used to calculate the microbial mass in sediments. Assuming a generic cell mass value of 2×10^{13} cells (g dry cells)$^{-1}$ and 100 μmol PLFA (g dry cell)$^{-1}$, therefore, 1 picomole PLFA is equivalent to 2.5×10^{4} cells.

The total viable biomass can be obtained from the total PLFA abundance. However, some of the fatty acids are from the deep-sea clams or from microalgae. Therefore, to estimate the microbial biomass, only bacterial fatty acids were considered (Findlay and Dobbs, 1993). The lists of bacterial fatty acids are shown in Table 16. The average total bacterial PLFA concentrations were 2.28×10^{4} and 1.83×10^{4} picomoles g^{-1} dry weight in the *Maorithyas* and *Calyptogena* sediments, which were about 12-15% of the total fatty acids for both communities (Table 17 and 18). The average microbial biomass was estimated to be 5.71×10^{8} and 4.58×10^{8} cells (g dry wt)$^{-1}$ for the *Maorithyas* and *Calyptogena* sediments, respectively.

Table 16. Two approaches for assigning PLFA to functional groups of microorganisms (Findlay and Dobbs, 1993)

A. A *Priori* approach	Fatty Acids
Functional Group	
Eukaryotes	C16:4ω1, C16:3, C18:4ω3, C18:3ω6, C18:2ω6, C18:3ω3, C20:4ω6, C20:5ω3, C22:5ω6, C22:6ω3
ω6 "animal" series	C18:3ω6, C18:2ω6, C20:4ω6, C22:5ω6
ω3 "plant" series	C18:3ω3, C20:5ω3, C22:6ω3
Eukaryotic photoautotrophs	C16:1ω13t, C18:3ω3, C18:1ω9
Bacteria	*i*C15:0, *a*C15:0, C15:0, *i*C17:0, *a*C17:0, C17:0, C18:1ω7c, C19:0cy(ω7,8), 10Me-C16:0, C17:0*cy*(ω7,8)
Desulfobacter	10Me-C16:0, C17:0*cy*(ω7,8)
Bacteria, anaerobic desaturase pathway	C18:1ω7c
Bacillus-type Gram-positive bacteria	*i*C15:0, *a*C15:0, *i*C17:0, *a*C17:0
B. A *Posteriori* approach	
Microeukaryotes	C16:4ω1, C16:3, C18:4ω3, C18:3ω3, C20:3ω6, C20:4ω6, C20:5ω3, C22:5ω6, C22:6ω3
Aerobic prokaryotes and eukaryotes	C16:1ω5, C16:1ω7c, C17:1ω6, C17:1ω9, C18:1ω7c, C18:1ω9, C18:2ω6
Gram-positive prok and other anaerobic bacteria (except 16:1ω13t)	C14:0, *a*C15:0, *i*C15:0, *i*C16:0, C16:1ω13t
SRB and other anaerobes	C16:0, 10Me-C16:0, *i*C17:0, *a*C17:0, C17:0*cy*, C17:0, C18:0, C19:0*cy*

* The position of the double bond is defined by the symbol "ω" followed by the number of carbons from the methyl end.

Table 17. Relative abundance, total PLFA concentration and estimated microbial biomass of different microbial functional groups in sediment samples from the *Maorithyas* colony.

		Maorithyas		
	Functional Group	B-74	C-018	Average
Relative	Bacteria (%)*	26%	11%	12%
Absolute	Total PLFA (picomole/g dry wt)	2.71×10^4	3.55×10^5	1.91×10^5
	Total bacterial PLFA* (picomole/g dry wt)	6.93×10^3	3.88×10^4	2.28×10^4
	Estimated bacteria biomass (cell/g dry wt)	1.73×10^8	9.69×10^8	5.71×10^8

*Relative proportions of reported biomarker fatty acids over total detectable fatty acids using Findlay and Dobbs (1993) functional group approach.

Table 18. Relative abundance, total PLFA concentration and estimated microbial biomass of different microbial functional groups in sediment samples from the *Calyptogena* colony.

		Calyptogena		
	Functional Group	374-2	C022	Average
Relative	Bacteria (%)*	15%	17%	15%
Absolute	Total PLFA (picomole/g dry wt)	1.55×10^5	8.23×10^4	1.19×10^5
	Total bacterial PLFA* (picomole/g dry wt)	2.26×10^4	1.41×10^4	1.83×10^4
	Estimated Microbial Biomass (cell/g dry wt)	5.64×10^8	3.52×10^8	4.58×10^8

*Relative proportions of reported biomarker fatty acids over total detectable fatty acids using Findlay and Dobbs (1993) functional group approach.

4. DISCUSSION

Deep-sea cold seeps comprise a complex chemosynthetic community functioning under high pressure, low temperature and sometimes limited nutrient supply (Guezennec and Fiala-Medioni, 1996). Therefore, it is difficult to study this unique environment by the conventional methods of bacterial analysis that require replicating deep-sea bacterial growth conditions such as pressure and temperature (Baird and White, 1985). In order to obtain quantitative data, lipid analysis is a more appropriate approach for the study of deep-sea cold seeps.

4.1. Microbial Biomass

Phospholipids can be used as a measure for viable cellular biomass (White et al., 1979). Despite the phospholipid approach's limitation – namely, that the PLFA concentration also reflects the environmental conditions to some extent (Green and Scow, 1999) – it has been shown to correlate well with microbial biomass measurement by other methods, such as muramic acid levels, total adenosine triphosphate or enzyme activities (Balkwill et al., 1988; Guezennec and Fiala-Medioni, 1996).

The total microbial biomass found in the *Maorithyas* and *Calyptogena* sediments was estimated at 5.71×10^8 and 4.58×10^8 cells (g dry wt)$^{-1}$, respectively. The microbial mass of the *Maorithyas* colony was 1.5 times more than the *Calyptogena* colony. The microbial biomass estimations of both colonies were about one magnitude lower than other cold seeps sites. The bacterial biomass of sediment from the Nankai Trough *Calyptogena* communities ranged from 7.9×10^9 to 8.8×10^8 cells (g dry wt)$^{-1}$ (Li et al., 1999). The biomass found in

the Barbados Trench sediment was about 10^9 cells (g dry wt)$^{-1}$. For these two studies at the Nankai Trough and the Barbados Trench, all fatty acids (except C16:1ω7 for the Nankai Trough study) were used to estimate the total microbial biomass. In this study, only bacterial fatty acids were included for microbial biomass estimation. It is expected that the total microbial biomass is slightly lower than the results obtained from the Nankai Trough and the Barbados Trench where fatty acids from clams were included in determining microbial biomass.

4.2 Microbial Community Structure of the Cold Seep Sediments

Fatty acid analysis can provide insight into the bacteria community structure (White et al., 1979). To distinguish the difference between two microbial communities, the functional-group approach can be used to interpret the phospholipid fatty acid profiles (Findlay and Dobbs, 1993). The functional approach consists of grouping suites of microorganism that share similar biochemical characteristics. The *a priori* and *a posteriori* approaches are the two methods to identify functional groups of microorganisms in the environmental samples. The *a posteriori* approach is one in which, typically, PLFA datasets are treated statistically and patterns of PLFA abundance in concentrations and/or proportions of change are analyzed to define the functional groups of microorganisms (Findlay and Dobbs, 1993). The *a priori* approach is one by which microbial functional groups are assessed based on the presence and/or absence of some particular PLFA biomarkers (Findlay and Dobbs, 1993; Findlay et al., 1990). It has been that suggested a holistic approach is needed to interpret PLFA profiles (Zelles, 1999). This is because many fatty acids are not

exclusive to specific microorganisms. Therefore, caution must be taken in using lipid biomarkers for microbial community analysis.

In this project, *a priori* approach was used to interpret the PLFA profiles of the sediments and clam organ samples. Phospholipid fatty acids are common in both bacteria and eukarya. Most bacterial fatty acids range from 10 to 20 carbons. They can be saturated, monounsaturated, polyunsaturated, branched and cyclopropyl (Goldfine, 1972; Kaneda, 1991). C16:0 and C18:0 fatty acids are the most abundant and widespread fatty acids. Nevertheless, some of the fatty acid biomarkers with methyl branching, double bonds or cyclic moieties are less common (Pancost and Damste, 2003). For examples, branched PLFA are common to Gram-positive bacteria (Harwood and Russell, 1984) and anaerobic Gram-negative bacteria (Parkes and Taylor, 1983; Ratledge and Wilkinson, 1988; Kaneda, 1991; White et al., 1996; Guezennec and Fiala-Medioni, 1996; Caudales et al., 1998). The 10-methyl branched fatty acid 10Me18:0 is a biomarker for *Actinomycetes* (Kroppenstedt, 1985, Kieft et al., 1994). Fatty acids with a cyclopropyl group are common in anaerobic bacteria, possibly sulfate reducers (Fang and Barcelona, 1998).

Sediments collected from the *Maorithyas* and *Calyptogena* communities exhibited similar fatty acid profiles that were dominated by large percentages of monounsaturated fatty acids ranging from 27% to 40% of the total fatty acids. It is suggested that both communities were predominated by Gram-negative bacteria. It has been reported that microalgae and some deep-sea symbiotic bivalves also produce ω7 series MUFA C16:$1^{\Delta 9}$ and C18:$1^{\Delta 11}$, as well as polyunsaturated fatty acids (Findlay, 1990; Ben Mlih et al., 1992; Jahnke et al., 1995; Guezennec and Fiala-Medioni, 1996). However, no or very little PUFA were detected from the *Maorithyas* and *Calyptogena* sediments. For this reason, the MUFA from two sediments

were mainly bacterial in origin (Guezennec and Fiala-Medioni, 1996). The presence of Gram-positive bacteria and Gram-negative anaerobic bacteria was suggested by the detection of terminal-branched fatty acids (Parkes and Taylor, 1983; Ratledge and Wilkinson, 1988; Kaneda, 1991; White et al., 1996; Guezennec and Fiala-Medioni, 1996; Caudales et al., 1998), but the total concentration of TBFA is less than that of MUFA.

4.2.1 Methanotrophic Bacteria

At cold seeps, complex chemosynthetic communities comprise a variety of microorganisms working in syntrophic partnerships. Syntrophic coupling between methanogens (operating in reverse) and sulfate reducing bacteria mediating AOM were supported by geochemical biomarkers and molecular biological data (Hinriichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001a,b; Michaelis et al., 2002; Joye et al., 2004). Even though studies indicate that methane-consuming archaea (ANME1 and ANME2) are responsible for the oxidation of methane (Hinrichs et al., 1999; Boetius et al., 2000; Valentine, 2002), biomarkers unique to methanotrophic bacteria ($C16{:}1^{\Delta 8}$, $C16{:}1^{\Delta 10}$ $C18{:}1^{\Delta 8}$ and $C18{:}1^{\Delta 10}$) are also present at the Barbados Trench cold seeps (Guezennec and Fiala-Medioni, 1996). However, these biomarkers are not present in the Japan Trench and the Nankai Trough cold-seeps (Li et al., 1999). This shows that methanotrophic Archaea may be the only methane consumers in the Japan Trench and Nankai Trough cold seeps instead of methanotrophic bacteria. This further suggests that microbial community structures of individual cold seeps may be different.

Hopanoids in sediment are almost always good indicators for organic matter's input from prokaryotes (Volkman, 2005). Hopanoids predominantly occur in aerobic bacteria, such

as methanotrophs, heterotrophs, and cyanobacteria. Several hopanoids were isolated from the Japan Trench sediments. The occurrence of hopanoids in the Japan Trench suggests that organic matter in the sediment was transported from the oxic zone to the deep sea. Two biological 17β(H),21β(H)-configuration hopanoids with 22R stereochemistry unique to living bacteria were isolated from the two communities' sediments (Thiel et al., 2003). This indicated that microorganisms capable of producing hopanoids were present in the Japan Trench cold seeps. Compound specific isotope analysis would provide further indications of the source of the hopanoids. If seeping methane was incorporated into organic compounds they would have extremely low $\delta^{13}C$ values (Hinrichs et al., 1999; Thiel et al., 2004).

4.2.2. Sulfate-reducing (SRB) and Sulfur-oxidizing Bacteria

Sulfate reduction at cold seeps is another vital process of AOM. Sulfate-reducing bacteria develop syntrophic interactions with methane-oxidizing microorganisms. SRB can utilize the reaction products of methane oxidation as electron donors to reduce sulfate in seawater to produce sulfide (Hoehler et al., 1994; Leveille and Juniper 2003). High sulfate reduction rates were observed in sediments beneath the bacterial mats or chemosynthetic macrofauna in the Sagami Bay cold seeps and the Gulf of Mexico cold seeps (Masuzawa et al., 1992; Joye et al., 2004).

The fatty acid composition of sulfate-reducing bacteria has been well studied (Taylor and Parkes, 1983, 1985; Edlund, 1985; Parkes and Calder, 1985; Dowling et al., 1986; Veinshtein et al., 1992; Parkes et al., 1993; Kohring et al., 1994; Lien et al., 1998; Konneke and Widdel, 2003; Elvert et al., 2003; Londry and Des Marais, 2004). The overall PLFA profiles collected from the Japan Trench cold seeps were very typical of sulfate reducing

bacteria (SRB) and other anaerobes. C16:0, 10MeC16:0, iC17:0, aC17:0, C17:0cy, C17:0 and C18:0 are biomarkers common to SRB (Findlay and Bobbs, 1993). The *iso* and *anteiso* C15:0 fatty acids can be attributed to *Desulfotomaculum* (Ueki and Suto, 1979; Liu et al., 1997; Kuever et al., 1999; Chang et al., 2001). The same TBFA fatty acids and MUFA $C16:1^{\Delta 9}$ and $C17:1^{\Delta 9}$ are also biomarkers for *Desulfococcus multivorans* (Rütters et al., 2002) whereas the MUFA $C16:1^{\Delta 9}$, $C17:1^{\Delta 9}$ and $C18:1^{\Delta 11}$ are biomarkers for *Desulfomicrobium sp.* Strain SAL (Rütters et al., 2002). The occurrence of biomarkers $C16:1^{\Delta 9}$c and C17:0*cy* in PLFA profiles, commonly associated with *Desulfomonile tiedjei*, suggest the presence of this bacterium. The detection of 10MeC16:0 suggests the presence of *Desulfobacter* and *Desulflobacterium*. It has been reported that 10MeC16:0 is also present in several other organisms, such as *Actinomycetes* (Kroppenstedt and Kutzner, 1978). However, *Actinomycetes* is also characterized by the high abundance of 10MeC18:0 (Dowling et al., 1986; Vainshrein et al., 1992), which is a fatty acid absent in both sediment lipid profiles. The detection of $C16:1^{\Delta 11}$t also suggests the presence of *Desulfobacter* as well as *Desulfomonas acetoxidans* (Dowling et al., 1986). The cyclopropyl fatty acid, C17:0cy, is also indicative for *Desulfobacter* (Konneke and Widdel, 2003; Londry et al. 2004).

Free-living sulfur-oxidizing bacteria and the endosymbiotic bacteria in the deep-sea clams play an important role in biogeochemical cycling of sulfur (Kato et al., 1999). They are able to oxidize sulfide, the product of AOM, to sulfate. Then the sulfate-reducing bacteria in the sediments can utilize the dissolved sulfate in seawater. The exceptionally high concentrations of MUFA $C16:1^{\Delta 9}$ and $C18:1^{\Delta 11}$ in the studied sediments suggest the possible contributions by sulfur-oxidizers (Larkin, 1980; Katayama-Fujimura et al., 1982; Jannasch, 1985; Jannasch et al., 1989; Guezennec and Fiala-Medioni, 1996). The high abundance of

C18:1$^{\Delta 11}$c and C16:1$^{\Delta 9}$ are common for the filamentous bacteria, *Beggiatoa* as well as *Thioploca* spp (McCaffrey et al., 1989; Grants, 1991). Biomarkers (C14:1, *i*C15:0, *a*C15:0, C16:1, C18:1) for *Thiomicrospira* thioautotrophs were detected from both communities sediments. *Thiomicrospira* were reported as one of the dual symbiotic bacteria of the *Maorithyas hadalis* clam (Fujiwara et al., 2001). The presence of thioautotrophic bacteria *Thiomicrospira* is indicated by the detection of unique biomarkers (C14:1, *i*C15:0, *a*C15:0, C16:1, and C18:1) from the *M. hadalis* gill tissue (Table 5) (Grimalt et al., 1992; Knittel et al., 2005). Therefore, the endosymbiotic bacteria of *M. hadalis* could be the source of *Thiomicrospira* biomarkers in *Maorithyas* sediment.

Glycolipids are another major membrane components of eukaryotes and bacteria. Glycolipids constitute a significant proportion of the membrane lipids in all photosynthetic organisms including cyanobacteria (Collins and Ferrier, 1995; Kim et al., 1999; Hiroyuki et al., 2000). Despite the high concentration of glycolipid fatty acids, no phytadienes (a major hydrocarbon of cyanobacteria derived from chlorophyll) were present in *Maorithyas* and *Calyptogena* (Zeng et al., 1992). Therefore, the source of glycolipid fatty acids might be from the deep sea instead of upper waters. They could be from the microorganisms and the macrofauna living at the cold seeps.

4.2.3. Microbial Community Structure of the *Maorithyas hadalis* and *Calyptogena phaseoliformis* Sediments

Complex microbial communities were present in both chemosynthetic communities. The linear relationship between the fatty acid concentrations of two chemosynthetic

communities can be described by the Pearson correlation coefficient. The Pearson coefficient between *Maorithyas* and *Calyptogena* sediment is 0.59, which means that the microbial structure of two different chemosynthetic communities are not identical. The total microbial biomass of the *Maorithyas* sediment is higher than that of the *Calyptogena* sediment, but the microbial structures are somewhat similar. Although the *C. phaseoliformis* and *M. hadalis* are deep-sea macrofauna that belong to different families, similarities were observed between the two organisms, (1) very high concentrations of sulfur in *M. hadalis* and *C. soyoae* gill tissue (Fujikura et al., 1999); (2) the bacterial symbionts in *C. phaseoliformis* and *M. hadalis* at the Japan Trench were coccid cells with diameters from 0.6 to 1.0 μm (Fiala-Medioni and Le Pennec, 1988, Fujikura et al., 1999); (3) the symbionts of *Calyptogena* and *Maorithyas* are members of the γ subdivision of the Proteobacteria (Fujikura et al., 1999; Kato et al., 1999). As a result, the symbiosis present in both organisms is comparable, possibly explaining the similar microbial community structure found in the two different communities in the Japan Trench. Therefore, this symbiosis is relatively common in the Japan Trench cold-seeps and can take place at different depths.

4.3. Organic Matter Input for the Japan Trench Cold-Seeps

Sterols and *n*-alkanes are good lipid indicators of organic input from marine and terrestrial environments. Sterols are tetracyclic triterpenoid lipid biomarkers that are usually found in terrestrial higher plants, marine algae and eukaryotes. However, some prokaryotes also synthesize sterols (Pearson et al., 2003, Volkman, 2005), including some methanotrophic bacteria such as *Methylococcus capsulatus* which synthesize 4-methyl sterols (Bird et al., 1971, Bouvier et al., 1976). Many studies have shown that sterols are also found

in cyanobacteria, although some analyses were based on inadequate analytical technique without the full recognition of the possibility of contamination (Volkman, 2005).

At the Japan Trench cold seep, deep-sea invertebrates are probably the source of the high concentrations of $C_{27}^{\Delta5}$ (cholest-5-en-3β-ol) and some other sterols (5α-cholestan-3β-ol, $C28^{\Delta5,22}$, 24-methylenecholest-5β-en-3ol, 24-methylcholest-5β-en-3-ol and 24-ethylcholest-5-en-3β-ol) in both communities (see next section). The sterol profiles of the cold seep sediments suggest that terrestrial plants are the major source of organic matter, because they usually have high amounts of C_{29} sterols (Volkman, 1986; Salt et al., 1991). Several microalgae sterols were isolated from the sediments. $C_{27}^{\Delta5}$, $C_{27}^{\Delta5,22}$, $C_{28}^{\Delta5,22}$ are common in diatoms and $C_{28}^{\Delta5,22}$ and $C_{29}^{\Delta5,22}$ are common in heptophytes. A few sterols from dinoflagellates were also found in the sediment, e.g., 5α-cholestan-3β-ol and 4α,23-trimethylcholest-22-en-3β-ol. These results suggest that microalgae, like diatoms and heptophytes, and dinoflagellates contribute part of the organic matter to the Japan Trench cold seeps.

It has been reported long chain *n*-alkanes (*n*-C_{21} to *n*-C_{36}), especially odd-numbered alkanes (C_{27}, C_{29} and C_{31}) in marine sediment are often used as biomarkers for terrestrial higher plants (Cranwell, 1973; Comet and Eglinton, 1987; Rieley et al., 1991; Ratnayake et al., 2005). High abundances of long chain *n*-alkanes found in the Japan Trench imply that terrestrial higher plants are contributing to the organic matter at the cold seeps. In contrast, marine bacterial and algal alkanes, which have short carbon chains (<C_{20}), were not found in either *Maorithyas* or *Calyptogena* colonies.

4.4 Symbiotic Bacteria in the *Maorithyas hadalis* Tissue

The fatty acids isolated from the bivalve can provide information about the symbionts living within the organism. It has been reported that the endosymbiotic bacteria of *M. hadalis* are living within the gill tissue (Fujiwara et al., 2001). Therefore, it is presumed that the leg tissue is bacteria-free. However, several TBFA (iC15:0, aC15:0, iC17:0) and one CYFA (C17:0cy) that are unique bacterial lipids were isolated from the phospholipid and glycolipid fractions of the *Maorithyas hadalis* leg samples. This suggests that either cross contamination took place when separating the clam tissue or some free-living bacteria in the sediment remained on the leg tissue. Therefore, caution must be made when identifying fatty acid biomarkers in the *M. hadalis* gill tissue. High concentrations of MUFA (C16:1, C16:$1^{\Delta 9}$, C 17:$1^{\Delta 11}$, C18:1, C18:$1^{\Delta 9}$, C20:$1^{\Delta 7}$ and C20:$1^{\Delta 13}$) and of PUFA (C18:2, C20:4, C20:5 and C22:2), which commonly found in deep-sea invertebrates (Jahnke et al., 1995), were detected from both phospholipid and glycolipid fractions of leg and gill tissue. Thus these fatty acids could be a good fatty acid biomarker for *M. hadalis*.

Several TBFA (*i*C15:0 and *i*C17:0) and CYFA (C17:0*cy* and C19:0*cy*) were isolated from the gill samples and an exceptionally high proportion of MUFA C16:$1^{\Delta 9}$ was detected in the gill tissue (75%) compared to the leg tissue (12%). This suggests that C16:$1^{\Delta 9}$, CYFA, and TBFA present in the gill is mainly from endosymbiotic bacteria. A high abundance of MUFA with either C16:$1^{\Delta 9}$ or C18:$1^{\Delta 11}$ predominating is an indicator for the presence of sulfur-oxidizing bacteria, especially C14:1, *i*C15:0, C16:1 and C18:1, which are common in the *Thiomicrospira* species (Grimalt et al., 1992; Knittel et al., 2005). These results corroborate the findings of Kato et al. (2001) that the symbionts found in *M. hadalis* are sulfur-oxidizing bacteria including the *Thiomicrospira* species. Furthermore, even though

hydrogen sulfide is a highly toxic substance for most organisms, large amounts of sulfur were detected from the hexane phase in the *Maorithyas hadalis* leg and gill tissues. This suggests that symbionts utilizing hydrogen sulfide are present in *Maorithyas hadalis* from the Japan Trench. It was found that all living *M. hadalis* in the Japan Trench were buried with about 20% of their shell exposed in a vertical position in the sediment (Fujikura et al., 1999). In this configuration, they can provide their endosymbiontic bacteria access to hydrogen sulfide produced by sulfate reduction which is taking place in the sediment just beneath the living *M. hadalis* clams.

The absence of hopanoids and 4-methyl sterols in the gill tissue is further confirmation that the symbiosis of *Maorithyas hadalis* is sulfur based and not methane based. The major sterol in clam tissue is cholesterol. Cholesterol is a very common compound found in animals. It was isolated from both leg and gill tissues. Five other sterols were present in leg but not gill tissue. So the question is whether the other five sterols were synthesized by *Maorithyas hadalis* or were from other sources. Since contamination of leg sample was observed based on the PLFA profiles, contamination could be one possible source of these five sterols. The absence of same sterols in gill tissue supports this suggestion; it is because gill should contain same set of sterol biomarkers.

5. CONCLUSIONS

5.1 Carbon and Sulfur Circulation

Lipid analysis of the Japan Trench cold-seeps sediments and of the *Maorithyas hadalis* tissues revealed the microbial structure of chemosynthetic communities, and the endosymbiotic bacteria living within the *Maorithyas hadalis* clam. Complex consortia of microorganisms are present in the Japan Trench sediments of *Maorithyas hadalis* and *Calyptogena phaseoliformis* communities. These include a variety of sulfate-reducing bacteria and sulfur-oxidizing bacteria. The total microbial biomass of *Maorithyas hadalis* sediment is higher than *Calyptogena phaseoliformis*. However, the microbial structures of the two communities are about the same. In addition, high concentrations of sulfur-oxidizing bacteria biomarkers were isolated from the clam tissue, suggesting that the endosymbiotic bacteria in *M. hadalis* are sulfur oxidizers and that they developed a sulfur-based symbiosis with the *M. hadalis* clam. A model of carbon and sulfur circulation in the Japan Trench chemosynthetic communities is proposed in Figure 19.

Anaerobic oxidation of methane (AOM) is an important biogeochemical process in the marine environments where methane is abundant, e.g., at seafloor cold seeps. AOM is mediated by syntrophic coupling between methane-oxidizing archaea and sulfate-reducing bacteria. Methanogens normally produce methane in anoxic environments. However, evidence shows that they can oxidize methane anaerobically in cold seeps (Hinrich et al., 1999). At the Japan Trench, methanogenic archaea (Hinrich et al., 1999), operating in reverse, oxidize the seeping methane to carbon dioxide and hydrogen or acetate. Then, a consortium of different sulfate-reducing bacteria species found in the Japan Trench utilize

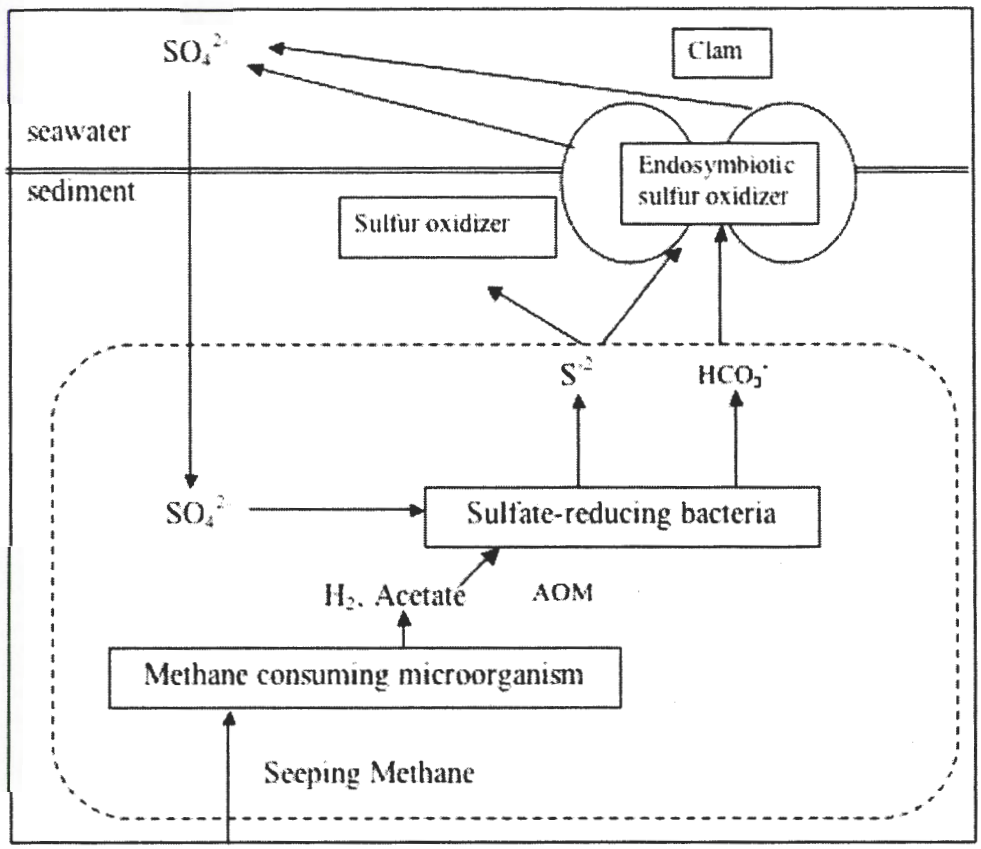

Figure 19. A model of carbon and sulfur circulation within the microbial communities and the chemosynthetic communities at the Japan Trench cold-seep environment.

the hydrogen or acetate and reduce the dissolved sulfate, leading to the high sulfate reduction rates which have been observed within the sediments (Masuzawa et al., 1992; Joye et al., 2004).

Chemosynthetic communities are another common feature of methane cold seeps. In the Japan Trench, both of the sulfur-based *Calytogena* and *Maorithyas* communities do not directly rely on the anaerobic oxidation of methane but on the hydrogen sulfide generated from the AOM. These deep-sea invertebrates do not have complete digestive systems but

contain autotrophic sulfur-oxidizing bacteria within their gill tissue. The hydrogen sulfide produced by sulfate reduction is either taken up by the macrofauna half buried in the sediment or utilized by free-living sulfur-oxidizing bacteria present in the sediment. Both endosymbiotic and free-living sulfur oxidizers are very important for the recycling of sulfur, because they can oxidize sulfide back to sulfate and support the growth of sulfate-reducing bacteria within the sediment. The Japan Trench communities are not totally independent of the contributions from photosynthesis, since a large amount of the organic matter input appears to come from the photic zone and terrestrial environment.

The high concentrations of sulfur found in *Maorithyas hadalis* suggest that *M. hadalis* has developed a sulfur-based symbiosis with their endosymbiotic bacteria. The hosts provide their endosymbionts access to sulfide. The endosymbiont in *Maorithyas hadalis* oxidizes the sulfide; at the same time they assimilate the organic compound either from sediment or from the upper water to make food for the deep-sea macrofauna.

5.2 Future Work

This project provides the background data on the total microbial biomass and structures of the chemosynthetic communities in the Japan Trench. Further analysis is necessary to provide additional insight in the anaerobic oxidation of methane there.

Recent work has highlighted the ubiquity of archaeal lipids in marine sediments (Hoefs et al., 1997; Schouten et al., 1998, 2000). Previous studies revealed that methane-consuming archaea at seeps play an important role in the anaerobic oxidation of methane (Hinrich et al., 1999) because they are the major consumers of methane at seeps. The membrane lipids of archaea are called phospholipid etherlipids. They are distinctly different

from bacteria or eukaryotes. The etherlipids' non-polar chains are linked by ether rather than ester linkage (Mancusi et al., 1985; Kates, 1993). Therefore, PLFA analysis is not able to detect archaeal lipids; phospholipid etherlipids (PLEL) analysis is required to study the archaeal community.

In addition, details about the carbon flow in the anaerobic oxidation process in the Japan Trench remain unknown. Compound specific isotope analysis (CSIA) of lipid biomarker analysis should provide more insight on the origins on the biomarkers and the biogeochemical processes operating in the sediments.

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